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**ADVANCES IN ENZYMOLOGY**  
**AND RELATED SUBJECTS OF**  
**BIOCHEMISTRY**  
**Volume VI**

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# ADVANCES IN ENZYMOLOGY

*AND RELATED SUBJECTS OF BIOCHEMISTRY*

**Edited by F. F. NORD**  
FORDHAM UNIVERSITY, NEW YORK, N. Y.

VOLUME VI

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## AFTER FIVE YEARS

When *Ergebnisse der Enzymforschung* became extinct, *Advances in Enzymology* was initiated as an outlet for the publication of critical reports abutting on the borderland between physiology, chemistry, microbiology and physical chemistry. The continued participation of this organ in keeping abreast of our vast subject has again resulted in a volume which, as has been the endeavor since its inception, approaches the true *polis* of the enzymologist.

It is also intended in the future to rotate and expand the subjects reported, at the same time limiting the articles to topics that are reasonably ready for critical discussion.

Volume VI is presented to the reader about one hundred years after separation from the living cell of the diastases by Payen and Persoz and of pepsin by Schwann. It is my sincere hope that the cessation of the armed hostilities will help to restore the community spirit and consolidate our efforts in our field in which the *Advances* have not as yet completed their mission.

THE EDITOR



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# THE BACTERIAL AMINO ACID DECARBOXYLASES

By

ERNEST F. GALE

*Cambridge, England*

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### I. Production of Amines by Bacteria

The production of amines as a result of bacterial putrefaction has been recognized since the beginning of the present century (9, 53), and Ellinger (19, 20) and Ackermann (1-4) showed that if media consisting of inorganic

salts, peptone, glucose, and certain amino acids are inoculated with putrefying material and the medium examined chemically after a period of some weeks, the amines corresponding to these amino acids can be isolated in fair yield. In this way the biological production of putrescine, histamine, cadaverine, tyramine,  $\beta$ -alanine,  $\gamma$ -aminobutyric acid, and  $\delta$ -aminovaleric acid from the corresponding amino acids was demonstrated. Pure strains of organisms were used for the production of histamine from histidine by Mellanby and Twort (51) and Berthelot and Bertrand (12). The organism isolated by the latter workers proved capable also of forming tyramine and tryptamine from tyrosine and tryptophan, respectively, and was accordingly named *Bacillus aminophilus intestinalis*. Other workers followed the formation of amines in culture by the growth of pure strains of various bacteria and found that *Escherichia coli* can produce histamine or tyramine (41, 42, 54), putrescine from arginine (5), and isoamylamine from leucine (6); *Bacillus mesentericus vulgatus* can form putrescine, cadaverine, and tryptamine (37), and many genera and species can form histamine (17, 41, 44, 46, 47, 51).

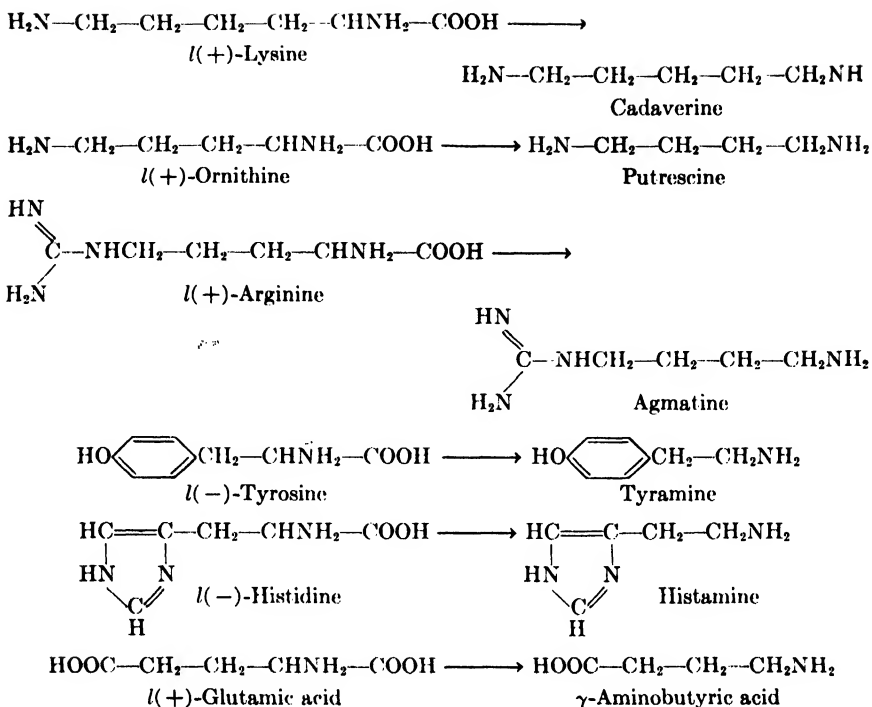
The greater part of this early work serves to emphasize the biological nature of amine production but gives little information concerning the mechanism of or the conditions necessary for such metabolism. It is noticeable that, in the majority of these cases, amines are produced only when fermentable carbohydrate is present in the growth medium. Koessler and Henke (48) carried out a detailed investigation of the formation of histamine by a "colon bacillus" using an extraction method to remove histamine from culture prior to colorimetric estimation. They showed that whenever the amine is produced, the medium first becomes distinctly acid and that "histamine is never formed except in the presence of an easily available source of carbon such as glycerol or glucose." The investigations were extended to other "colon bacilli" (41, 42) and the same general conclusion was reached. It was found that some of these organisms were able to form tyramine when grown in a medium containing tyrosine and fermentable carbohydrate, but that the organisms which produce tyramine would not produce histamine and vice versa. These authors made the further interesting discovery that those organisms which attacked tyrosine to form tyramine when the growth medium was allowed to become acid, also attacked the amino acid in an alkaline medium, but the product was now phenol and not tyramine. Eggerth and co-workers (18) improved the methods of extraction and estimation of histamine in cultures and studied (17) the formation of histamine by many strains of various genera including *E. coli*, *Salmonella*, *Eberthella*, *Aerobacter*, *Clostridium welchii*, etc. The organisms were grown in various determined media containing inorganic salts, glucose, and, in some cases, asparagine or peptone to assist growth. In positive cases, histamine formation from histidine began within 24 hours and continued for 4 to 5 days. Experiments were carried out in which the pH of the media was controlled and it was found that histamine production was most rapid when the medium pH lay between 5.0 and 5.5 and was markedly, in some cases completely, inhibited by medium pH values higher than 6.5. With some strains it was found that temperature affects the histamine formation, organisms such as certain

strains of *E. coli* and *Aerobacter aerogenes* having optimal temperatures below 30° C. while others, such as *Cl. welchii*, displaying optimal histamine production at growth temperatures around 37°.

Gale (24-27) showed that amines are produced by bacteria through the action of specific amino acid decarboxylases and that these enzymes are formed within bacterial cells in response to certain well-defined conditions of growth. The enzymes have been obtained in a cell-free condition (21, 22, 32, 65) and their properties have now been investigated in detail. The present article summarizes the knowledge that has been gained of the nature, action, formation, and distribution of these bacterial amino acid decarboxylases.

## II. Decarboxylation of Amino Acids by Bacteria

The simple decarboxylation of an amino acid results in the formation of the corresponding amine with the liberation of carbon dioxide. If the reaction takes place at an acid pH value, the carbon dioxide is evolved in the gas phase and the course of the reaction can be followed manometrically. The bacterial amino acid decarboxylases, so far identified, all react at optimum pH values lying 2.5 to 6.0, so that when washed suspensions of bacteria possessing such enzymes are shaken with the appropriate amino acid substrate in a manometer, amine production is indicated by an evolution of carbon dioxide. During recent years many bacterial genera and species have been tested under suitable experimental conditions for their power to decarboxylate the common amino acids; so far only six amino acids have been attacked in such experiments (see Scheme 1). These six amino acids have one property in common: that, in addition to the terminal —COOH group and the  $\alpha$ -NH<sub>2</sub> group, they all have a further polar group situated at the end of the molecule removed from the —COOH group attacked. That the integrity of this third polar group is necessary for decarboxylation to occur is indicated by the fact that if the group is substituted or altered, then the enzyme can no longer attack the molecule concerned. Thus *l*(+)-lysine decarboxylase cannot attack lysine in which either NH<sub>2</sub> group has been methylated or benzoylated; *l*(-)-tyrosine decarboxylase cannot attack tyrosine in which the —OH group has been methylated or benzoylated and is inactive toward phenylalanine; *l*(+)-glutamic acid decarboxylase cannot attack any peptide containing glutamic acid whether the peptide linkage attaches to the 1 or 5 position. In each case the enzyme concerned is specific for the natural isomer of the amino acid substrate. Substitution of the  $\alpha$ -NH<sub>2</sub> group results in complete



SCHEME 1

inhibition of the attack and the corresponding keto acids are not attacked. Since *l*(+)-lysine decarboxylase cannot decarboxylate *l*(+)-ornithine, the length of the carbon chain between the polar groups is also of importance.

These findings, considered in conjunction with the fact that no monoaminomonocarboxylic acid is attacked in this way, suggest that for an amino acid to evoke the formation of a decarboxylase in bacteria it must possess: (1) a free COOH group in the 1 position; (2) a free  $\alpha$ -NH<sub>2</sub> group; (3) a free terminal group of polar nature; and (4) the natural *levo* configuration.

It would seem that, for an enzyme to produce the strain necessary to result in the splitting off of carbon dioxide from the —COOH group of the substrate molecule, there must be a two-point attachment between the substrate and the enzyme protein, and the presence of polar groups in the right stereochemical relation is necessary for the evocation of the enzymes during growth.

The question arises whether the reverse condition holds true: can any

amino acid with two polar groups other than the  $\text{—COOH}$  group evoke the formation of a decarboxylase in some organism or other? If this should be the case, then we should expect to find bacteria which will attack aspartic acid or tryptophan. Virtanen and Laine (69) have claimed that *Rhizobium leguminosarum* decarboxylates aspartic acid with the formation of  $\beta$ -alanine, while Ackermann (1-4), in the course of his studies on the production of amines during putrefaction, isolated  $\beta$ -alanine from media containing aspartic acid, so that it is probable that bacteria do occur which will carry out this decarboxylation. There are also claims in the literature (12, 37) that tryptamine has been obtained by the action of bacteria on tryptophan, but studies in this unit involving a survey of some 200 coliform organisms, 800 streptococci, 30 clostridia, and representative strains of *Pseudomonas*, *Proteus*, *Bacilli* (including *B. mesentericus*, *B. subtilis*, etc.), etc., have so far not yielded any organism which can decarboxylate any common amino acid other than the six listed above. All these studies have involved the use of washed suspensions and a manometric technique of following decarboxylation and it may be that very slow decarboxylations, which would yield a product over a long period of incubation in a growth experiment, would not appear significant in the washed suspension investigations.

The activities of amino acid decarboxylases quoted in this paper are expressed as  $Q_{\text{CO}_2} = \mu\text{l. CO}_2$  liberated from substrate at  $30^\circ \text{C.}$  and at the optimum pH per hr. per mg. dry weight of organism.

### III. Conditions Necessary for Formation of Amino Acid Decarboxylases in Bacteria

For the formation of active amino acid decarboxylases in bacteria the following conditions have to be fulfilled:

1. The organism concerned must possess such enzymes in its potential enzymic constitution.
2. Growth must take place in the presence of the specific substrate.
3. The organism must be capable of synthesizing codecarboxylase or, if the organism cannot accomplish this synthesis, the growth medium must contain certain factors involved in the formation of codecarboxylase.
4. The growth medium must be acid.
5. With some organisms, amino acid decarboxylases are formed to a significant extent only if growth occurs at temperatures lower than  $30^\circ \text{C.}$
6. The enzymes are fully developed within the organism only at the end of active cell division.

These conditions will now be considered in detail.



### 1. *Distribution of Amino Acid Decarboxylases in Potential Enzymic Constitution of Bacteria*

The potential enzymic constitution of an organism is the sum total of all the enzymes that that particular organism can produce when grown under suitable conditions and represents the enzymic repertoire of the organism from which the actual enzymic constitution of any particular cell or culture is selected by the conditions of growth and formation of that cell or culture (28). All bacteria cannot produce all six known decarboxylases; in fact it is probable that the majority of bacteria are unable to produce these enzymes at all. In recent years the author and his colleagues have investigated the distribution of the six amino acid decarboxylases among the potential enzymic constitutions of various bacterial genera and species. The main results can be summarized as follows.

***Escherichia coli* and Related Organisms.**—Most coliform organisms when grown under suitable conditions can form amino acid decarboxylases. Of 151 strains of *E. coli* investigated, 114 have possessed arginine decarboxylase, 142 lysine decarboxylase, 130 glutamic acid decarboxylase, 14 histidine decarboxylase (a larger number may have possessed this enzyme in a weakly active state but generally organisms whose activity ( $Q_{CO_2}$ ) has been less than 2 to 3 have been recorded as negative), and 6 strains have possessed tyrosine decarboxylase. Many of these organisms have not been tested against ornithine, but of those tested, 90% have possessed ornithine decarboxylase. No organism has been found which possesses all six decarboxylases but any number from 5 to 0 may occur in specific strains. Tyrosine decarboxylase occurs rarely among coliform organisms. Stadler and Neus (63) found that 9 out of 100 strains of *E. coli* possessed this enzyme and it has also been recorded in this group by Hanke and Koessler (42). The finding of these last workers that the tyrosine and histidine enzymes are not found together appears to be confirmed. No one enzyme ever occurs in constant association with any other. The activities of the enzymes when the cells are grown and tested under optimal conditions varies anywhere between  $Q_{CO_2} = 1$  and 1200, being of the order 200 to 300 for the arginine, lysine, and glutamic acid enzymes in most cases, and less than 100 for histidine decarboxylase. Other organisms having similar distributions of these enzymes are *Aerobacter aerogenes* and *Klebsiella pneumoniae*.

**Streptococci.**—Some 800 strains of streptococci have been examined for decarboxylase activity. Approximately 500 strains of *S. faecalis* or streptococci belonging to Lancefield's group D have possessed tyrosine decarboxylase to a variable degree, but no other amino acid decarboxylase.

Representative strains of streptococci belonging to Lancefield's groups A to F have all shown tyrosine decarboxylase activity, but no other. A limited number of *S. lactis* strains have been tested and have shown no decarboxylase activities. The activities of the tyrosine enzymes vary widely with strain, the majority of *S. faecalis* strains from normal healthy infants having  $Q_{CO_2}$  values less than 50, while organisms isolated from certain diarrheal conditions have  $Q_{CO_2}$  values 200 to 300.

**Clostridia.**—Of ten strains of *Cl. welchii* type A examined, nine possessed histidine decarboxylase and glutamic acid decarboxylase, while the classical strain S.R.12 possessed only glutamic acid decarboxylase. Strains of *Cl. welchii* types B, C, and D possessed both decarboxylases. Four strains of *Cl. septicum* possessed ornithine decarboxylase only and this enzyme was present in a highly active state in the four organisms. Two strains of *Cl. bifermentans* possessed glutamic acid decarboxylase, one of two strains of *Cl. fallax* possessed histidine decarboxylase, and *Cl. aerofœtidum* had both tyrosine and glutamic acid enzymes. Representative strains of many other species of *Clostridia* (*sporogenes*, *histolyticum*, *chauvoei*, *oedematiens*, *tetani*, *butyricum*, etc.) were inactive.

**Proteus.**—Some twenty strains of *Proteus vulgaris*, *morganii*, etc., have been tested. The majority possess both glutamic acid decarboxylase and ornithine decarboxylase but some strains may possess either or none.

**Bacilli.**—No detailed investigation has as yet been undertaken but preliminary investigations show that *B. subtilis* and *B. mesentericus* strains possess weak arginine, lysine, ornithine, and glutamic acid enzymes.

**General.**—No active enzymes have been found in organisms belonging to *Staphylococcus* or *Pseudomonas*. *Lactobacilli* have not yet been examined. In all cases tests have been made on the whole range of common amino acids (24) but in no case, so far, has any organism been discovered which could decarboxylate any amino acid other than the six listed above, under the experimental conditions used.

## 2. Adaptation to Substrate

Table I shows the activity toward the six amino acids of various strains of *E. coli*, grown in a simple salt-glucose-ammonia medium. When these organisms are grown in such a medium free of amino acids, the amino acid decarboxylases, with the exception of the glutamic acid enzyme, are not produced. The addition of the amino acid substrate to the growth medium results in the production of the corresponding decarboxylase (provided that this is present in the potential enzymic constitution of the organism tested). Thus the presence of 1% lysine in the growth medium results

in the production of an organism having a  $Q_{CO_2}$  (lysine) of 210 compared with the value of 194 for the same organism growing in the fully nutrient casein digest-glucose medium. In the same way the addition of 1% ornithine to the growth medium results in the adaptive formation of ornithine decarboxylase. Similar results are obtained with the tyrosine and histidine enzymes. Arginine decarboxylase formation apparently requires factors other than simple substrate adaptation as the addition of 1% arginine to the growth medium results in an organism having a  $Q_{CO_2}$  (arginine) of 27 compared with the value of 330 when growth takes place in the digest medium. The glutamic acid decarboxylase is formed to some extent in the simple amino acid-free medium, but the activity is doubled by the addition of specific substrate during growth. There is adaptive formation of all these enzymes in response to the presence of the specific substrate during growth but the full development of the arginine decarboxylase apparently requires other factors.

TABLE I  
ADAPTIVE FORMATION OF AMINO ACID DECARBOXYLASES\* IN *Escherichia coli*†

Decarboxylase	<i>E. coli</i> strain	Additions to medium							
		None	Lysine	Arginine	Ornithine	Glutamate	Histidine	Tyrosine	Casein digest
l(+)-Lysine	86	4	210	..	4	..	..	..	194
l(+)-Arginine	86	0	..	27	..	..	..	..	330
l(+)-Ornithine	86	3	..	..	225	..	..	..	145
l(+)-Glutamic acid	TY	45	..	..	..	88	..	..	100
l(-)-Histidine	86	0	..	..	..	..	7	..	18
l(-)-Tyrosine	HE	0	..	..	..	..	..	60	63

\* Growth medium: inorganic salt mixture, including  $(NH_4)_2HPO_4$ , + 2% glucose + additions (1%) as above.

† Activities expressed in values of  $Q_{CO_2}$  at 30° C. and optimum pH.

### 3. Codecarboxylase Factors in Growth Medium

Gale (25) found that strains of *Streptococcus faecalis* possess a very active tyrosine decarboxylase but that, when these organisms are grown in a simplified medium, the activity of the resulting organisms is greatly decreased. For example, a strain of *S. faecalis* grown in casein digest-glucose has a  $Q_{CO_2}$  (tyrosine) of 218, but when grown in a salt-glucose-marmite medium the activity is 8 and is raised to 20 by the addition of 1% tyrosine to the growth medium. There was adaptation to substrate in this case, but the resulting activity was considerably less than that developed in the complex casein digest medium. It was suggested (28)

that the organism is unable to synthesize some factor involved in enzyme production other than the substrate. Bellamy and Gunsalus (10) followed up this suggestion and tested the effect of the addition of various vitamin B factors on the activity of *S. faecalis* growing in a medium consisting of hydrolyzed gelatin, potassium phosphate, glucose, yeast extract, tyrosine, tryptophan, and cystine. Table II shows the effect of adding eight growth

TABLE II

ACTION OF THE PRESENCE OF B VITAMINS IN GROWTH MEDIUM ON THE TYROSINE DECARBOXYLASE ACTIVITY OF *S. faecalis* (10)

Medium	$Q_{CO_2}^N$ (tyrosine)
Basal (see text).....	77
Basal + eight factors as below.....	890
Basal + factors without thiamin.....	650
riboflavin.....	550
pyridoxine.....	210
pantothenate.....	800
nicotinic acid.....	130
<i>p</i> -aminobenzoic acid.....	780
biotin.....	640
"folie acid".....	680
Basal + pyridoxine (100 $\mu$ g./100 ml.) + nicotinic acid (500 $\mu$ g./100 ml.).....	900
Fully nutrient medium.....	2500

factors to this medium and then of the omission of these factors one at a time. The  $Q_{CO_2}$  values are calculated on a nitrogen basis and the values are correspondingly higher than others calculated on a dry weight basis and quoted in this paper. The addition of the eight growth factors results in a marked enhancement of the tyrosine decarboxylase activity, although the activity is still considerably below that recorded for growth in a fully nutrient medium (10). Omission of each of the factors, in turn, shows that all have some effect, possibly within experimental error in some cases, but that the omission of either pyridoxine or nicotinic acid has a marked effect on the activity, while the addition of these two factors together has an enhancing effect equal to that of the complex of eight factors. The development of the full activity in this organism is therefore dependent upon the presence of pyridoxine and nicotinic acid in the growth medium. The addition of these two factors to the simplified medium used for the growth of *E. coli* will not increase the adaptive formation of arginine decarboxylase by that organism.

4. *pH of Growth Medium*

In general, when bacteria are grown in an alkaline medium, they attack amino acids by deamination, and when growth occurs in an acid medium, by decarboxylation (31). This is correlated with a change in the ionization of the amino acid substrate and with a change in the enzymic constitution of the organism with the *pH* of the growth environment. If bacteria are grown in casein digest medium, free from fermentable carbohydrate, adjusted to various *pH* values within the growth range, and the organisms are harvested and their activities tested for amino acid decarboxylases within their potential enzymic constitution, we find that the enzymes are not formed when growth takes place at an alkaline *pH* and that the lower the *pH*, on the acid side, during growth, the greater the formation of the decarboxylases (24, 25, 27).

TABLE III

RELATION BETWEEN AMINO ACID DECARBOXYLASE ACTIVITY AND *pH* OF THE MEDIUM DURING GROWTH\*

Decarboxylase	Organism	<i>pH</i> of medium during growth				
		8.5	8.0	7.0	6.0	5.0
l(+)-Ornithine	<i>E. coli</i>	0	10	80	310	980
	<i>Cl. septicum</i>	..	80	220	380	680
l(-)-Tyrosine	<i>S. faecalis</i>	0	15	250	530	670
	<i>Cl. aerofetidum</i>	0	15	38	48	57
l(+)-Lysine	<i>E. coli</i>	2	30	118	250	410
	<i>E. coli</i> †	..	..	218	426	721
l(+)-Arginine	<i>E. coli</i>	0	3	20	70	120
l(+)-Glutamic acid	<i>E. coli</i>	15	18	51	75	89
	<i>Cl. bifermentans</i>	0	15	95	110	130
l(-)-Histidine	<i>E. coli</i>	0	0	1	3	33
	<i>Cl. welchii</i>	0	1	4	8	28

\* Activities expressed as  $\text{QCO}_2 = \mu\text{l. CO}_2$  liberated from substrate per hr. per mg. dry weight of bacteria, measured at 30° C. and at the optimum *pH* in each case.

† Cells disintegrated by drying in acetone, ether, and air.

Table III shows the activity of various decarboxylases of various organisms grown at environmental *pH* values between 8.5 and 5.0. That the activity figures really represent an increase in the enzyme content of the cells with acid growth conditions has been shown in the case of the lysine decarboxylase of *E. coli* by disintegrating the cells by acetone treatment and measuring the activity of the extracts (values marked with †). It is a common finding that such disintegration of the cells results in an increased activity (16, 21, 32). Since the enzymes are formed only during growth in the presence of their substrates and at an acid *pH*, it follows that

the formation of the enzymes is strictly adaptive to the presence of the specific substrate in the form with an unionized  $\text{—COOH}$  group.

The enzymes are formed to the greatest extent when growth takes place near the limit of acid tolerance for the organism concerned, but the crop formed under such conditions is small. For large-scale work it is more satisfactory to grow the organism in a medium containing glucose or other fermentable carbohydrate; the fermentation of the carbohydrate during growth greatly increases the crop and, at the same time, causes a fall of pH, with the result that a heavy crop of active organisms is produced. The presence of the carbohydrate appears to have no effect on activity that cannot be attributed to the change in pH resulting from fermentation (24).

### 5. Effect of Growth Temperature

Eggerth (17) noted that the production of histamine by some organisms is greater when growth takes place at low temperatures than at the usual temperature of  $37^\circ\text{C}$ . Gale (24) found that the production of amino acid decarboxylases by strains of *E. coli* is inhibited by growth temperatures of the order of  $37^\circ$  and that optimum formation of the enzymes takes place when growth occurs at  $20\text{--}26^\circ$ . Table IV shows that the effect is again due to a difference in the enzyme content of the cells since similar differ-

TABLE IV  
EFFECT OF GROWTH TEMPERATURE ON THE FORMATION OF AMINO ACID DECARBOXYLASES  
IN *E. coli*\*

Decarboxylase	Preparation	Growth temperature		
		$20^\circ\text{C}$ .	$26^\circ\text{C}$ .	$37^\circ\text{C}$ .
<i>l</i> (+)-Arginine	Washed cells	...	240	128
<i>l</i> (-)-Histidine	Washed cells	...	18	12
<i>l</i> (+)-Ornithine	Washed cells	...	90	8
<i>l</i> (+)-Lysine	Washed cells	252	205	55
<i>l</i> (+)-Lysine	Acetone-dried cells	421	337	156

\* Activities expressed as  $Q_{\text{CO}_2} = \mu\text{l. CO}_2$  liberated per hr. per mg. dry weight of preparation.

ences in activity with growth temperature are obtained whether the activity measurements are made with intact or acetone-dried cells. This temperature effect is characteristic of the organism rather than of the enzyme, for amino acid decarboxylases are formed to a greater extent at  $37^\circ$  than at  $25^\circ$  in organisms such as *S. faecalis* or *Cl. welchii* (25, 27).

## 6. Age of Culture

When growth takes place under optimal conditions the formation of amino acid decarboxylases does not take place until fairly late in the growth period. Cultures harvested within ten hours (at 25° C.) or four hours (at 37° C.) of inoculation have little or no decarboxylase activity whatever the medium pH, and the activity develops as growth proceeds, becoming optimal at about the time that active cell division ceases (24). This is illustrated for various decarboxylases in three organisms in Table V.

TABLE V  
EFFECT OF AGE OF CULTURE ON DEVELOPMENT OF AMINO ACID DECARBOXYLASES  
IN GROWING BACTERIA\*

Age of culture, hrs.	Decarboxylase substrate				
	Arginine	Lysine	Glutamic acid	Tyrosine	Histidine
4	...	...	...	...	2
6	4	5	6	50	13
8	2	10	5	103	26
10	6	30	4	143	28
12	70	153	27	286	27
14	105	282	50	325	28
16	110	316	75	330	..
18	103	310	73	286	..
Growth ceases at	16 hrs.	16 hrs.	16 hrs.	15 hrs.	10 hrs.
Growth temp., ° C.	25°	25°	25°	37°	37°
Organism	<i>Escherichia coli</i>			<i>S. faecalis</i>	<i>Cl. welchii</i>

\* Growth medium = tryptic digest of casein + 2% glucose. Organisms harvested at times indicated and activities determined with washed suspensions at 30° and optimum pH. Activities expressed as  $Q_{CO_2}$  =  $\mu$ l. CO<sub>2</sub> liberated per hr. per mg. dry weight of organism.

When growth takes place in the presence of carbohydrate, the delay in the appearance of the enzymes within the cells can be partly, but not wholly, explained by the necessity for the development of a suitable degree of acidity in the medium, but similar effects are obtained in any case in the absence of carbohydrate (27).

*General.*—For cultivation of active organisms on a large scale, growth has been carried out in a casein digest containing 2% glucose (with the addition of 0.1% marmite for growth of streptococci) at 25° for coliform organisms or 37° for streptococci and clostridia, and growth has been continued until active cell division ceases before organisms were harvested.

## IV. Preparation of Cell-Free Amino Acid Decarboxylases

With the exception of ornithine decarboxylase, the enzymes are unaffected by acetone drying of the bacterial cells containing them. The

enzymes can then be extracted from the dried cells by incubation with buffer solutions. The lysine, arginine, glutamic acid, and histidine enzymes can be extracted from appropriate preparations with borate buffer at pH 8.5 and the tyrosine enzyme can be extracted from streptococcal powders with acetate buffer at pH 5.5. The cell debris can be spun off leaving an opalescent liquid containing the active enzyme (21, 22, 32, 65). The enzymes can then be purified by adsorption on to and elution from alumina C $\gamma$  [lysine and histidine decarboxylases (22, 32)] or from calcium phosphate [tyrosine enzyme (21)], followed by fractionation with ammonium sulfate solutions as indicated in Table VI. In this way lysine decarboxylase has been purified 90 times to a final activity at 30° of  $Q_{CO_2}^C = 46,000$ ; tyrosine decarboxylase 116 times to  $Q_{CO_2}^C = 46,300$ ; and histidine decarboxylase 52 times to  $Q_{CO_2}^C = 3670$ .

TABLE VI

METHODS USED FOR THE PARTIAL PURIFICATION OF THE *l*(+)-LYSINE, *l*(-)-TYROSINE, AND *l*(-)-HISTIDINE DECARBOXYLASES (21, 22, 32)

Decarboxylase substrate:	<i>l</i> (+)-Lysine		<i>l</i> (-)-Tyrosine	<i>l</i> (-)-Histidine
Organism used as source:	<i>E. coli</i>	<i>B. cadaveris</i>	<i>S. faecalis</i>	<i>Cl. welchii</i> B.W. 21
Treatment	$Q_{CO_2}^{C*}$	$Q_{CO_2}^C$	$Q_{CO_2}^C$	$Q_{CO_2}^C$
(1) Washed cell suspension	508	963	400	71
(2) Acetone-powder suspension	860	1,830	800	145
(3) Crude extract from (2)	1,850	2,170	1,690	254
(4) Eluate from				
(a) alumina C $\gamma$	4,100	4,050	.....	577
(b) calcium phosphate	.....	.....	5,040	...
(5) Precipitated with ammonium sulfate	4,210	4,500	6,220	715
(6) First ammonium sulfate fractionation:				
(a) 40-56% satd. (NH $_4$ ) $_2$ SO $_4$	21,100	7,650	.....	...
(b) 50-63%	.....	.....	18,400	...
(c) 50-67%	.....	.....	.....	3,670
(7) Second fractionation (of 6)				
(a) 40-47% satd. (NH $_4$ ) $_2$ SO $_4$	41,300	17,500	.....	...
(b) 53-58%	.....	.....	45,700	...
(8) Third fractionation (of 7)				
41-47% satd. (NH $_4$ ) $_2$ SO $_4$	46,000	33,500	.....	...
(9) Repetition of stage 4	.....	.....	46,300	...
Purification achieved	90	35	116	52

\*  $Q_{CO_2}^C = \mu\text{l. CO}_2$  liberated from substrate per hr. per mg. carbon of preparation. Highest activities obtained in each case indicated by italicized figures.

The ornithine decarboxylase will not stand acetone drying and loses 90-95% of its activity when organisms containing it are so treated. A cell-free preparation of orni-



thine decarboxylase has been achieved by disintegrating a thick active suspension of *Cl. septicum* by shaking with minute glass beads in a machine constructed from the directions given by Curran and Evans (15). After 2 hours of shaking, the majority of the cells are disintegrated and can be centrifuged down leaving the active enzyme in the opalescent supernatant. It has not been possible to purify this enzyme any further because it is very unstable.

One of the objects of purifying the enzymes in a cell-free state is to obtain specific preparations of each. In some cases this can be done by choosing, as the starting material, an organism specific for the decarboxylation of one amino acid only. Thus, *Cl. welchii* S.R.12 decarboxylates *l*(+)-glutamic acid only; *Cl. septicum* Pasteur is specific for *l*(+)-ornithine; *S. faecalis* for *l*(-)-tyrosine; and a strain of *E. coli* has been isolated which is specific for *l*(+)-arginine. *l*(+)-Lysine decarboxylase is found in many coliform organisms in association with other decarboxylases, but the method of purification worked out by Gale and Epps (32) starting with *Bacterium cadaveris* results in a specific enzyme preparation. The organism most active toward *l*(-)-histidine is *Cl. welchii* but the enzyme is accompanied in this organism by glutamic acid decarboxylase; the preparation of *l*(-)-histidine decarboxylase from *Cl. welchii* B.W.21 results in the destruction of the glutamic acid enzyme and the production of a preparation specific for the decarboxylation of histidine (22).

## V. Properties of Purified Amino Acid Decarboxylases

### 1. Specificity

The distribution of the six amino acid decarboxylases among the potential enzymic constitutions of bacteria indicates that six enzymes, each specific for one amino acid, are involved. Six specific preparations have now been worked out (21, 22, 30, 32, 65) and in each case the preparation will catalyze the decarboxylation of a single amino acid only. Thus *l*(+)-lysine decarboxylase will not attack *d*(-)-lysine or  $\alpha$ -methyl-,  $\epsilon$ -methyl-,  $\alpha$ -acetyl-, or  $\epsilon$ -acetyl-*l*(+)-lysine (32); *l*(-)-tyrosine decarboxylase will not attack *d*(+)-tyrosine, *l*(-)-phenylalanine, *dl*-serine, *l*-tyrosine sulfonic acid, N-methyl-*dl*-tyrosine, methoxy-*l*-tyrosine, N-methyl-methoxy-*l*-tyrosine, *l*-thyroxine, *l*-thyronine, or glycylyltyrosine (21); *l*(-)-histidine decarboxylase will not attack *d*(+)-histidine,  $\beta$ -alanyl-histidine, acetyl- or benzoylhistidine, thiohistidine or any histidine peptide (22, 34); *l*(+)-glutamic acid decarboxylase will not attack *d*(-)-glutamic acid, N-methylglutamic acid,  $\alpha$ -ketoglutaric acid, *l*(-)-aspartic acid, or glutathione (30). These examples demonstrate that the enzyme is specific

in each case for the natural isomer of the complete unsubstituted amino acid substrate.

The substitution of a hydroxy group in the substrate molecule, other than in any of the essential polar groups, does not interfere with decarboxylation. Thus *l*(+)-lysine decarboxylase can decarboxylate *l*(+)-hydroxylysine; *l*(-)-tyrosine decarboxylase can decarboxylate *l*-3,4-dihydroxyphenylalanine ("dopa"), and *l*(+)-glutamic acid decarboxylase can decarboxylate synthetic  $\beta$ -hydroxy-*l*-glutamic acid. In each case the rate of decarboxylation of the hydroxy derivative is less than that of the unsubstituted amino acid substrate (21, 22, 32).

The decarboxylation is quantitative and manometric estimation of the carbon dioxide evolved from the substrate in the presence of the specific enzyme can be used for the estimation of the substrate. The method can be applied to the estimation of amino acids in protein hydrolyzates (30). Table VII gives some typical results obtained for the analysis of protein hydrolyzates by the decarboxylase method with results obtained by other methods and workers quoted for comparison. In every case there is agreement within experimental error: these results provide a further demonstration of the specificity of the decarboxylase preparations used.

TABLE VII

SPECIFICITY OF AMINO ACID DECARBOXYLASE PREPARATIONS: ANALYSIS OF PROTEIN HYDROLYZATES (30)\*

Amino acid	Protein	Analysis by specific decarboxylase method	Analysis by other methods and references
<i>l</i> (-)-Histidine	Edestin	3.66	3.50 (50)
	Hemoglobin	12.51	12.56 (68)
<i>l</i> (+)-Lysine	Edestin	2.44	2.44 (50)
	Hemoglobin	10.47	9.4 (68)
<i>l</i> (-)-Tyrosine	Edestin	1.83	1.85 (49)
	Hemoglobin	1.41	1.43 (23)
<i>l</i> (+)-Arginine	Edestin	27.5	28.7 (50)
	Hemoglobin	6.84	6.95 (50)
<i>l</i> (+)-Glutamic acid	Edestin	10.06	10.04 (8)
	Hemoglobin	4.42	3.76 (8)
<i>l</i> (+)-Ornithine	Edestin	Nil	Nil
	Tyrocidine	13.11	13.2 (36)

\* Amino acid contents of protein hydrolyzates expressed as per cent total nitrogen.

## 2. *pH* of Optimum Activity

The amino acid decarboxylases are active over a narrow range of *pH* with optima in all cases lying between *pH* 2.5 and 6.0. Table VIII lists the *pH* optima determined for the various enzymes as investigated (a)

in the intact cell and (b) in purified preparations. The  $pH$  optima found for the activity measured with intact cells are found to vary from culture to culture and with the composition of the growth medium, but in most cases are found to have a value more acid than the corresponding values found with cell-free preparations. Thus the  $pH$  optimum of  $l(-)$ -histidine decarboxylase in washed suspensions of *Clostridium welchii* varies between 2.5 and 3.0 (27), but the value determined with the purified preparation is steady at  $pH$  4.5 (22); similarly, the  $pH$  optimum for  $l(+)$ -arginine decarboxylase in washed suspensions of *Escherichia coli* varies between 4.0 and 4.8, while the steady value for the cell-free preparation is 5.2 (65). Decarboxylation at an acid  $pH$  results in the formation of an alkaline amine and the evolution of carbon dioxide with a consequent shift of the environmental  $pH$  toward neutrality. It is suggested later that one of the functions of the amino acid decarboxylases is to act as a neutralization mechanism in an unfavorably acid medium, and it may be that this difference between the  $pH$  optima of the enzymes as measured in intact cells and in cell-free preparations is some reflection of the difference between the  $pH$  values of the internal and external environment of the cell as a result of the decarboxylase action.

### 3. Effect of Substrate Concentration

Table VIII also lists the Michaelis constants of the cell-free preparations compared with the apparent Michaelis constants measured with washed

TABLE VIII  
PROPERTIES OF THE AMINO ACID DECARBOXYLASES

Decarboxylase	$pH$ optimum of		Michaelis constant of	
	Intact cell preparation	Cell-free preparation	Intact cell preparation	Cell-free preparation
$l(-)$ -Histidine	2.5-3.0	4.5	0.00075 <i>M</i>	0.00075 <i>M</i>
$l(+)$ -Lysine	4.5-5.0	6.0	0.0028	0.0015
$l(+)$ -Arginine	4.0-4.8	5.2	0.00056	0.00075
$l(+)$ -Glutamic acid	4.0-4.5	4.5	0.005	0.027
$l(+)$ -Ornithine	5.0-5.5	5.2	0.003	0.004
$l(-)$ -Tyrosine	5.0-5.5	5.5	.....	.....

suspensions of organisms as source of enzyme. The affinities of the enzymes differ widely from that of  $l(+)$ -arginine decarboxylase (Michaelis constant = 0.00075 *M*) to that of  $l(+)$ -glutamic acid decarboxylase (Michaelis constant = 0.027 *M*) and only in the latter case is there any marked difference between the affinity of cell-free preparation and of the intact

organism preparation of the enzyme. In the case of *l*(+)-glutamic acid decarboxylase there is a marked decrease in the affinity following extraction of the enzyme from the cell, suggesting that the enzyme undergoes some damage during the preparative process. It is not possible to obtain values for the Michaelis constant of the tyrosine enzyme as the enzyme is not saturated by saturated solutions of tyrosine.

#### 4. Quantitative Nature of the Decarboxylation

Estimation of the carbon dioxide evolved during the decarboxylation of the six amino acids by the six decarboxylase preparations shows that, when allowance is made for carbon dioxide retention, gas output represents 96–98% theoretical (30). The quantitative nature of the decarboxylation is confirmed by the analytical figures quoted above in Table VII. If racemic mixtures of the amino acid substrate are used, then the carbon dioxide output corresponds to 45–49% theoretical (21, 22, 32) and the preparations can be used as a method for the resolution of such racemic mixtures and the preparation of the unnatural isomer (52).

#### 5. Action of Inhibitors

Table IX summarizes the action of common inhibitors on the action of the six amino acid decarboxylases (65). The enzymes are all sensitive to the presence of *silver* ions, the tyrosine and glutamic acid enzymes being considerably less sensitive than the others. All the enzymes are sensitive to *mercury* ions, the tyrosine enzyme again being the least sensitive. With the exception of the ornithine decarboxylase, which is completely inhibited by  $M \times 10^{-5}$  copper, the decarboxylases are not abnormally sensitive to the presence of *copper* ions and none has any marked sensitivity to *iron* ions. All the enzymes are inactivated by *potassium permanganate*, ornithine decarboxylase being particularly sensitive, a fact which may be correlated with the sensitivity of this enzyme to copper.

All six decarboxylases are sensitive to *cyanide* and it has been shown in the case of the lysine and arginine enzymes that this inhibition is reversible (32). This suggests the presence of a metal in the enzyme constitution although the enzymes, other than lysine decarboxylase, are not markedly sensitive to *sodium azide*. Lysine decarboxylase is 95% inhibited by 0.001 *M* hydrocyanic acid or sodium azide, but is not affected by carbon monoxide, hydrogen sulfide, sulfanilamide, or the "copper inhibitors" (32, 64); spectrometric examination of ashed purified enzyme preparations failed to show the presence of any metals in significant amounts (32).

The cyanide sensitivity may be due to the presence of an aldehyde group in the constitution of some of the enzymes (see below).

The significance of the sensitivity of some of the enzymes to keto fixatives such as *hydroxylamine*, *hydrazine*, and *semicarbazide* will be discussed below.

TABLE IX

EFFECT OF INHIBITORS ON ACTIVITY OF CELL-FREE AMINO ACID DECARBOXYLASES (65) \*

Inhibitor	pI	Amino acid decarboxylase					
		Lysine	Tyrosine	Arginine	Ornithine	Histidine	Glutamic acid
AgNO <sub>3</sub>	5	98	...	...	98	13	4
	4	100	...	35	100	100	20
	3	100	39	100	100	100	81
	2	100	99	100	100	100	100
HgCl <sub>2</sub>	5	98	...	14	...	...	...
	4	100	...	100	35	58	27
	3	100	...	100	100	94	100
	2	100	97	100	100	100	100
CuSO <sub>4</sub>	6	...	...	...	27	...	...
	5	...	...	...	100	...	...
	4	46	...	7	100	...	4
	3	89	98	51	100	61	10
FeSO <sub>4</sub>	2	98	100	100	100	92	33
	4	30	...	...	16	...	...
	3	82	26	23	32	...	0
	2	100	66	54	67	15	0
KMnO <sub>4</sub>	5	10	24	...	53	...	6
	4	100	100	17	98	15	41
	3	100	100	100	100	94	100
	2	100	100	100	100	94	100
KCN	4	81	...	22	40	...	93
	3	93	64	98	77	20	100
	2	100	93	100	96	97	100
	1	...	...	...	...	...	...
NaN <sub>3</sub>	4	61	...	7	36	...	...
	3	98	...	21	51	...	10
	2	100	0	100	97	0	27
	1	...	...	...	...	...	...
NH <sub>2</sub> .NH <sub>2</sub>	5	95	...	62	80	...	...
	4	100	30	71	92	...	0
	3	100	98	95	100	...	13
	2	100	100	100	100	10	43
NH <sub>2</sub> OH	5	95	43	91	50	...	7
	4	100	97	95	98	54	42
	3	100	100	100	100	100	96
	2	100	100	100	100	100	96
Semicarbazide	6	42	...	...	...	0	...
	5	76	90	45	77	33	0
	4	98	98	78	97	52	3
	3	100	100	94	100	77	6
Sulfanilamide	2	100	100	100	100	89	24
	1	...	...	...	...	...	...
	0	...	...	...	...	...	...
	-1	...	...	...	...	...	...
Codecarboxylase present in enzyme	2	15	9	21	35	51	0
	1	...	...	...	...	...	...
	0	...	...	...	...	...	...
	-1	...	...	...	...	...	...
		+	+	+	+	-	-

\* pI = negative logarithm of molar concentration of inhibitor. Inhibition expressed as percentage.

Sulfanilamide has a marked effect on histidine decarboxylase and this inhibition appears to be specific for the complete sulfanilamide molecule as neither N<sup>4</sup>- nor N<sup>1</sup>-substituted sulfonamides are active as inhibitors (22).

Certain of the enzymes, particularly the ornithine and lysine enzymes, are inhibited by the presence of high concentrations of salts such as ammonium sulfate or sodium sulfate (32, 65). The effect is reversible and is not apparently related to a coenzyme dissociation (65).

Common inhibitors which have no significant effect on the decarboxylases in concentrations less than 0.01 *M* are iodoacetate, fluoride, urethan, chloramine T, 8-hydroxyquinoline, sulfathiazole, etc.

## VI. Resolution of Enzymes into Apoenzymes and Coenzyme Moieties

Precipitation of *l*(+)-lysine decarboxylase with ammonium sulfate at an alkaline *pH* yields a protein which, on solution, is inactive toward lysine but which can be reactivated by the addition of boiled preparations of enzyme, bacteria, yeast, liver, etc. This suggests that the lysine decarboxylase dissociates at an alkaline *pH* into specific protein apoenzyme and coenzyme moieties with the result that precipitation of the protein with ammonium sulfate results in a coenzyme-free apoenzyme preparation. It is not possible to prepare the apoenzyme by dialysis against distilled water or ammonium sulfate solutions, as such treatment results in inactivation of the protein. The most satisfactory method of preparation is to precipitate the enzyme twice with 66% saturated ammonium sulfate solution containing 10% by volume of 0.880 ammonia solution. Table X shows that the protein so precipitated can be reactivated toward lysine by the addition of coenzyme (codecarboxylase) prepared as described below. The double precipitation with ammoniacal ammonium sulfate does not always result in complete resolution of the enzyme but this occurs if the preparation is kept in solution for a few days in the ice chest (32).

The *l*(-)-tyrosine decarboxylase spontaneously dissociates to a certain extent during purification (21) and consequently must be tested in the presence of excess coenzyme for activity determinations. The dissociation can be completed either by two consecutive precipitations with 66% saturated ammonium sulfate containing 3% by volume of 0.880 ammonia, or by standing the enzyme solution in the ice chest for 72 hours followed by dialysis overnight against glass-distilled water. In either case the resulting preparation is activated toward tyrosine by the addition of codecarboxylase preparations.

The precipitation of the lysine and tyrosine decarboxylases by am-

moniacal ammonium sulfate results in the irreversible inactivation of some of the enzyme—amounting to about 50% in the case of the tyrosine enzyme and 60–70% of the lysine enzyme. Preparations of the arginine decarboxylase are more difficult to resolve in this manner but an arginine apodecarboxylase preparation has been made by repeated precipitation of the protein with ammonium sulfate solutions containing progressively larger amounts of ammonia solution (65). The preparation was activated toward arginine by the addition of codecarboxylase preparations. Ornithine decarboxylase is irreversibly inactivated by ammonium sulfate precipitation but solutions of the enzyme dissociate spontaneously on standing; the dissociation is presumably followed by decomposition (probably oxidation) of the coenzyme as the activity of the preparations rapidly decreases on standing, but can be restored by the addition of codecarboxylase preparations (65).

The apoenzyme preparations of any one of these four enzymes can be activated toward their substrates by the addition of a boiled preparation of any one of the untreated enzymes, thus indicating that the four enzyme preparations contain a substance or substances that will function as coenzyme or coenzymes to the four apoenzymes.

It has not been possible to resolve or prepare apoenzymes of either the histidine or the glutamic acid decarboxylases.\* In both cases the enzyme preparations have been submitted to precipitation by ammoniacal ammonium sulfate of various strengths, to dialysis against distilled water or ammonium sulfate solutions, to prolonged standing at 0° C., to precipitation at acid pH values, etc., and in no case has any reversible resolution been achieved (22, 65). Further, boiled preparations of either histidine decarboxylase or glutamic decarboxylase will not act as a source of coenzyme for tyrosine or lysine apodecarboxylases. This suggests that these two enzymes differ from the remaining four decarboxylases in that they do not possess codecarboxylase as part of the enzyme structure (22, 65).

In Table IX the enzymes are grouped according to whether they have been demonstrated to contain codecarboxylase or not. The survey of the action of inhibitors was undertaken in order to find out whether it is possible to differentiate between codecarboxylase enzymes and noncodecarboxylase enzymes by sensitivity to inhibitors (65). Inspection of Table IX shows that two groups of inhibitors differentiate between the two types of decarboxylase. First, although none of the enzymes is markedly sensitive

\* Umbreit and Gunsalus (65a) claim to have effected a partial resolution of glutamic decarboxylase from a strain of *E. coli* by dialysis for 24 hrs. at pH 2 and 0° C. The activity of the preparation was increased by addition of either pyridoxal phosphate or codecarboxylase concentrate.

TABLE X  
REVERSIBLE RESOLUTION OF AMINO ACID DECARBOXYLASES

Decarboxylase	Treatment	μl. CO <sub>2</sub> liberated per 5 min.	
		Alone	Plus codecarboxylase
l(+)-Lysine	Twice pptd. with 66% satd. ammonium sulfate containing 10% ammonia (sp. gr. = 0.880)	20	195
l(-)-Tyrosine	Dialysis against glass-distd. water	10	174
l(+)-Arginine	As for lysine enzyme	20	118
l(+)-Ornithine	Standing 48 hrs. at 0° C.	25	110
l(-)-Histidine	No resolution achieved	..	..
l(+)-Glutamic acid	No resolution achieved	..	..

to iron, the four codecarboxylase enzymes are markedly inhibited by 0.01 *M* Fe<sup>++</sup>, which has no significant inhibitory action on either the histidine or the glutamic acid decarboxylase. Second, the group of keto fixatives are all more inhibitory toward the codecarboxylase enzymes than toward the other two enzymes. *Hydrazine* in a concentration of 0.001 *M* produces 95–100% inhibition of the codecarboxylase enzymes, but no inhibition of histidine decarboxylase (which is not completely inhibited by 0.1 *M* hydrazine), and 13% inhibition of the glutamic acid enzyme. *Hydroxylamine* in 0.0001 *M* concentration produces 95–100% inhibition of the codecarboxylase enzymes, 54% inhibition of histidine decarboxylase, and 42% inhibition of glutamic acid decarboxylase. *Semicarbazide* in 0.001 *M* concentration produces insignificant inhibition of the glutamic acid enzyme, 77% inhibition of the histidine enzyme, but 94–100% inhibition of the other four enzymes which, with the exception of the arginine enzyme, are 97% inhibited by 0.0001 *M* semicarbazide. These results indicate that the codecarboxylase enzymes possess a keto or aldehyde group which is essential for enzymic activity.

## VII. Distribution, Properties, and Preparation of Codecarboxylase

### 1. Distribution

The apoenzyme preparations of the lysine, tyrosine, arginine, and ornithine decarboxylases are activated toward their substrates by the addition of a source of codecarboxylase to the reaction mixture. Consequently, the apoenzymes can be used for a test for the presence of codecarboxylase since, if a suitable preparation of any substance will produce an activation



of the apoenzyme toward its substrate, it is highly probable that that substance contains codecarboxylase or a closely related product. Further, since the rate of decarboxylation of substrate is roughly proportional to the amount of codecarboxylase added to a given amount of apoenzyme, as long as the enzyme is not saturated with coenzyme (see Fig. 1, page 27), it is possible to assay the codecarboxylase content of tissues, etc., on a comparative basis. This has been done with a number of cells using *l*(+)-lysine apodecarboxylase and adding amounts of boiled, disintegrated cells

TABLE XI  
DISTRIBUTION OF CODECARBOXYLASE\* (33)

Tissue	Units codecarboxylase per mg. C
Rat skeletal muscle.....	1.54†
<i>Bacillus subtilis</i> .....	1.5
<i>Staphylococcus aureus</i> .....	1.48
Rat liver.....	1.22
<i>Escherichia coli</i> .....	1.12
Brewers' yeast.....	1.03
<i>Pseudomonas aeruginosa</i> .....	0.765
Rat kidney.....	0.63
<i>Clostridium welchii</i> .....	0.465
<i>Sarcina lutea</i> .....	0.41
Rat heart.....	0.385†
Rat lung.....	0.38
<i>Saccharomyces cerevisiae</i> .....	0.34
<i>Brassica oleracea</i> (wild cabbage).....	0.29†
<i>Pisum sativum</i> (garden pea).....	0.24†
Rat gut.....	0.23†
Rat brain.....	0.21
<i>Streptococcus faecalis</i> .....	0.13
Cow milk.....	0.043
Rat blood.....	0.037†
Human urine.....	Nil

\* Codecarboxylase content expressed as units of lysine codecarboxylase per mg. carbon of tissue (33).

† Sampling errors make these analyses approximate only.

so that, for a given amount of enzyme, the amount of codecarboxylase added in each case was never more than sufficient to produce a rate of decarboxylation half that of the saturated enzyme (33). Table XI gives the results found for various cells and quoted as units of lysine codecarboxylase per milligram carbon of tissue; the figures have no absolute values but serve to show the comparative codecarboxylase contents of the tissues. It is obvious that codecarboxylase has a wide distribution, being found in all the living cells investigated whether of bacteria (irrespective of amine

production), yeasts, or animal or plant tissues. If the tissues are assayed with regard to the codecarboxylase of tyrosine apodecarboxylase it is found, with one or two insignificant exceptions, that the distribution of the codecarboxylase is qualitatively and quantitatively the same whichever apoenzyme is used for assay purposes.

The following substances have been tested for codecarboxylase activity with negative results: coenzyme I, coenzyme II, thiamin, thiamin monophosphate, thiamin diphosphate, riboflavin, riboflavin phosphate, riboflavin-adenine-dinucleotide, adenylic acid, adenine, adenosine, inosine, hypoxanthine, glutathione, yeast nucleic acid, nicotinic acid, nicotinic amide, uracil, biotin, pantothenic acid, pyridoxine, *p*-aminobenzoic acid, inositol, thiochrome, xanthopterin, "folic acid," pyridoxylic acid, and various mixtures of these substances.

## 2. Properties

Using apoenzyme preparations as a method of assaying codecarboxylase, it is possible to investigate the properties of the coenzyme. For these studies a codecarboxylase preparation worked up from dried brewers' yeast has been used (33).

**Salts.**—Codecarboxylase forms salts with metals. The *silver*, *mercury*, and *lead* salts are soluble in acid and precipitated at neutrality; the active substance cannot be regenerated from these precipitates by hydrogen sulfide as the codecarboxylase then becomes adsorbed on the metallic sulfides and no satisfactory method of elution has been discovered. The *barium* salt is soluble in water at all *pH* values tested but is precipitated by 60% ethanol or methanol at *pH* 7.0. Codecarboxylase is precipitated by *phosphotungstic acid*, but not by *picric* or *picrolonic* acids.

**Solubility in Organic Solvents.**—Codecarboxylase cannot be extracted from acid, neutral, or alkaline solutions into ether; it can be extracted from acid solution into *n*-butanol, isobutanol, phenol, or cresols, but not from alkaline solution; the free acid is soluble in ethanol or methanol.

**Stabilities.**—Purified codecarboxylase preparations are rapidly inactivated by boiling in weak acid, two hours in 0.1 *N* sulfuric acid at 100° C. resulting in 90% inactivation; on the other hand, they are remarkably stable to alkali, no inactivation being produced by four hours in normal or 0.1 *N* sodium hydroxide at 100°. Purified preparations are unstable in air at *pH* 7.0 undergoing oxidation or hydrolysis at room temperature. Treatment of codecarboxylase with nitrous acid, hypobromous acid, or benzoyl chloride has no effect on activity.

### 3. Preparation of Codecarboxylase Concentrate

A method of concentration of natural codecarboxylase has been worked out on the basis of these properties (33). Convenient starting material is dried yeast (brewers' or bakers') which is extracted at 37° C. with 0.75% baryta and the sediment then centrifuged down, leaving the bulk of the active material in the supernatant fluid. The centrifugate is then purified as in Scheme 2.

	DEGREE OF PURIFICATION
<i>Dried yeast</i> .....	1
↓ Extract with baryta at room temperature	
<i>Centrifugate</i> .....	2
↓ Precipitate with two volumes methanol at pH 7.0	
<i>Filtrate</i>	
↓ Precipitate with barium acetate	
<i>Precipitate</i>	
↓ Wash exhaustively with hot water	
↓ Decompose in normal sulfuric acid.....	75
↓ Extract with liquid phenol	
<i>Mother liquor</i>	
↓ Saturate with ammonium sulfate	
↓ Extract with liquid phenol	
<i>Phenol extract</i>	
↓ Extract with water and ether mixture	
<i>Water phase</i> .....	255
↓ Acidify to pH 1 with nitric acid	
↓ Extract with isobutanol	
<i>Isobutanol extract</i>	
↓ Extract with sodium hydroxide	
<i>Alkaline extract</i>	
↓ Neutralize with nitric acid.....	675
↓ Add lead acetate	
<i>Lead precipitate</i>	
↓ Decompose in sulfuric acid.....	2,500
↓ Neutralize with excess barium acetate	
<i>Centrifugate</i>	
↓ Add one volume ethanol	
<i>Barium precipitate</i> .....	4,300
↓ Decompose in sulfuric acid	
↓ Add lead acetate to pH 5	
<i>Centrifugate</i>	
↓ Add lead acetate in excess	
↓ Neutralize with dilute sodium hydroxide.....	15,000
<b>LEAD PRECIPITATE</b>	

SCHEME 2

The final lead precipitate can be dried *in vacuo* and kept indefinitely, being regenerated for use by decomposition in dilute sulfuric acid. It is a yellow-white amorphous powder which gives a faintly yellow solution on decomposition. The composition of the lead salt is not constant and preparations vary considerably in their activities. The stage of the preparation which is least satisfactory is the phenol extraction after saturation of the mother liquor with ammonium sulfate and attempts to increase the scale of the preparation above laboratory scale have been found to result in less active preparations, mainly as a result of inactivation occurring at this stage. Elementary analysis of one

lead salt preparation gave: carbon, 16.0; hydrogen, 2.3; nitrogen, 3.6; phosphorus, 1.0, and lead, 55.5%.

The preparation, freed from lead, is highly active as codecarboxylase for the apoenzymes of lysine, arginine, ornithine, or tyrosine decarboxylases. During preparation the degree of purification achieved at each stage was assayed against both the lysine and the tyrosine enzymes (33) and the increase in purity over each step was the same, within experimental error, whichever enzyme was used for the assay. There is, therefore, little doubt that these two enzymes have the same coenzyme.

The rate of decarboxylation of substrate by the apoenzyme is dependent upon the concentration of codecarboxylase present in the reaction mixture and the curves relating rate of decarboxylation to codecarboxylase concentration are, in all cases, typical of those obtained for an apoenzyme-coenzyme dissociation (see Fig. 1, page 27). In the case of *l*(+)-lysine decarboxylase, the enzyme is half-saturated with coenzyme when an amount of the purified concentrate containing 0.06  $\mu$ g. carbon per ml. is added to the system (21, 32, 65).

It is certain that the lead concentrate prepared as above is not a pure preparation of codecarboxylase. It will be shown below that the coenzyme is probably pyridoxal phosphate, the lead salt of which would contain 7.4% phosphorus so that the concentrate may not contain more than about 13% codecarboxylase. Attempts to isolate an aldehyde derivative from the lead salt have so far yielded a small amount of a 2,4-dinitrophenyl-semicarbazone, which gives a positive test for the presence of phosphate.

### VIII. Chemical Nature of Codecarboxylase

**Tyrosine Decarboxylase Activity of Pyridoxine-Deficient Streptococci.**—Bellamy and Gunsalus (10) showed that, when a strain of *Streptococcus faecalis* with potential tyrosine decarboxylase activity is grown in a simplified medium (page 8), growth takes place, but the resulting organisms have negligible tyrosine decarboxylase activity. They found that the organisms produce the enzyme only if the growth medium contains, in addition to tyrosine, sufficient amounts of pyridoxine and nicotinic acid. Since the organism is nutritionally exacting toward pyridoxine, it follows that the development of the enzyme requires amounts of pyridoxine in excess of simple growth requirements. Snell and others have shown that there is a factor in animal tissues which will replace pyridoxine in the nutrition of certain lactic bacteria and when assayed against *S. lactis* R is more effective than pyridoxine itself; this factor has

been called "pseudopyridoxine" (61). If pyridoxine is treated in various ways as, for example, autoclaving with cystine (56), treatment with hydrogen peroxide (14), etc., its activity increases as a growth factor for *S. lactis* R, suggesting that pseudopyridoxine may be an oxidation product of pyridoxine. *S. lactis* R, which is apparently a strain of *S. faecalis* (40), will grow in a medium in which pyridoxine is replaced by alanine (60) and the cells are then inactive toward tyrosine but can be activated by the addition of pseudopyridoxine preparations made from pyridoxine, the stimulation of the tyrosine decarboxylase system by the preparations being in proportion to their pseudopyridoxine content (11). This presumably means that when the streptococci are grown in a pyridoxine-deficient medium, they are able to synthesize the protein moiety of tyrosine decarboxylase, but not the coenzyme or prosthetic group moiety.

**Nature of "Pseudopyridoxine."**—Following the indications obtained from microbiological assay concerning the nature of pseudopyridoxine, Harris, Heyl, and Folkers (43) synthesized certain pyridoxine derivatives including pyridoxal (with an aldehyde group in position 4) and pyridoxamine (with an amine group in position 4). Both these derivatives are active as the pseudopyridoxine growth factor for *S. lactis* R (57). Snell (62) finds that pyridoxal has 5000–8000 times, and pyridoxamine 6000–9000 times the activity of pyridoxine as growth factor for *S. lactis* R although the comparative activities are less for other organisms, and all three compounds have approximately the same activity as growth factors for *Saccharomyces cerevisiae*; Snell suggests that the biologically active substance is either pyridoxal or pyridoxamine and that these figures result from the varying ability of organisms to synthesize the aldehyde or amine derivative from pyridoxine. He has produced indirect evidence that pyridoxine, pyridoxal, and pyridoxamine all exist naturally in varying amounts in various tissues (58).

**Pyridoxal as Missing Factor in Tyrosine Decarboxylase Activity of Pyridoxine-Deficient Streptococci.**—Once the pyridoxine derivatives were available, they were tested for their ability to activate pyridoxine-deficient streptococci toward the decarboxylation of tyrosine (38); washed suspensions of such organisms were activated by pyridoxal but not by pyridoxamine or pyridoxine itself. Using the washed suspensions of streptococci as apoenzyme preparation, the half-saturation concentration of pyridoxal corresponds to 0.15  $\mu$ g. per 3 ml. giving an apparent dissociation constant for the enzyme-coenzyme complex =  $3 \times 10^{-7}$  molecules per liter. However if the cells are dried, then pyridoxal is no longer effective as activator unless adenosine triphosphate is also added or, alterna-

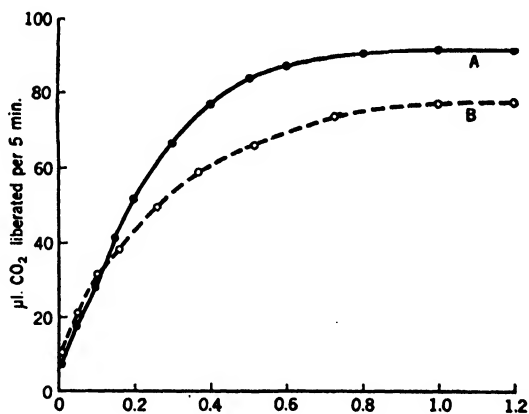
tively, the pyridoxal is phosphorylated by treatment first with thionyl chloride and then by silver dihydrogen phosphate (39).

**Codecarboxylase Function of Pyridoxal Phosphate.**—The results obtained by Bellamy and Gunsalus suggest strongly that a phosphorylated

TABLE XII  
CODECARBOXYLASE ACTIVITY OF "PYRIDOXAL PHOSPHATE" (7)

Apodecarboxylase	$\mu\text{l. CO}_2$ liberated from substrate per 5 min.		
	Alone	Plus codecarboxylase	Plus pyridoxal phosphate
<i>l</i> (-)-Tyrosine	2	110	116
<i>l</i> (+)-Lysine	15	106	104
<i>l</i> (+)-Arginine	15	70	75
<i>l</i> (+)-Ornithine	29	78	74

derivative of pyridoxal is the coenzyme of tyrosine decarboxylase. This has been confirmed by Baddiley and Gale (7) with the apoenzyme preparation of tyrosine decarboxylase. Table XII shows the activation of the



(A)  $\mu\text{g. carbon}$  of codecarboxylase preparation per 3 ml.

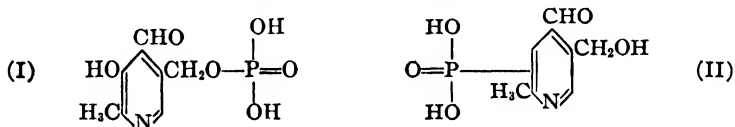
(B) ml. pyridoxal phosphate preparation per 3 ml.,  $\times 10$ .

Fig. 1.—Rate of decarboxylation of *l*(+)-lysine by the apoenzyme of *l*(+)-lysine decarboxylase in the presence of codecarboxylase and "pyridoxal phosphate" preparations. Contents of cups: 0.25 ml. apoenzyme solution (preparation A or B); 0.5 ml. *M*/15 *l*(+) lysine; 2.0 ml. *M*/5 phosphate buffer, pH 6.0; codecarboxylase or "pyridoxal phosphate" preparation as above + water to 0.25 ml. Temperature, 30° C.

apoenzymes of the lysine, tyrosine, arginine, and ornithine decarboxylases toward their substrates by the addition of excess of either codecarboxylase

concentrate or of a phosphorylated pyridoxal preparation. Figure 1 shows the variation of the rate of decarboxylation of lysine in the presence of lysine apodecarboxylase and increasing amounts of codecarboxylase concentrate on the one hand and of "pyridoxal phosphate" on the other.

**Identity of Codecarboxylase and "Pyridoxal Phosphate."**—Up to the time of writing neither the natural codecarboxylase from yeast nor the synthetic pyridoxal phosphate has been obtained in a pure state.\* The structure of the active substance in pyridoxal phosphate has not been satisfactorily determined, since both groups of workers have used preparations consisting of pyridoxal treated with thionyl chloride followed by silver dihydrogen phosphate. The evidence suggests that the active material consists of an orthophosphate of pyridoxal in which the phosphate group is on the primary hydroxyl group (as in I):



but it is possible that the phosphoryl residue is on the phenolic group (as in II). The properties of the active material in this synthetic preparation are similar to those determined for the natural codecarboxylase, and Table XIII shows that the stabilities of the two active substances toward acid and alkali are also similar. The presence of the aldehyde

TABLE XIII  
STABILITIES OF CODECARBOXYLASE AND PYRIDOXAL PHOSPHATE (7)†

Treatment	Codecarboxylase, units per ml.	Pyridoxal phosphate, units per ml.
Initial untreated	32	23
After 1 hr. at 100° C. in		
0.1 N NaOH	31	24
N NaOH	31	20
0.1 N H <sub>2</sub> SO <sub>4</sub>	11	7

† Activity estimated against tyrosine apodecarboxylase and expressed as units codecarboxylase per ml. of preparation in each case.

\* Gunsalus, Umbreit, Bellamy, and Foust (40a) describe the preparation of a purified synthetic product as its barium salt. The preparation contains 6.2% organic phosphorus, about 50% barium, and 32% pyridoxal estimated from the ultraviolet spectrum; the phosphorus content thus corresponds to one phosphate radical per mole of pyridoxal, which is in agreement with the above formula. Further data in this paper suggest that the phosphoryl residue in the synthetic product is on the phenolic group and not on the primary hydroxyl group.

group in the coenzyme explains the sensitivity of the codecarboxylase enzymes to keto or aldehyde monoxide fixatives (see Table IX).

**Identity of Coenzyme for the Four Decarboxylases.**—It has been assumed that the lysine, tyrosine, arginine, and ornithine decarboxylases have the same coenzyme. As none of the enzymes has been obtained in a crystalline state or tested, so far, with pure preparations of coenzyme, this has not been definitely proved but the assumption is based upon the following evidence:

1. The distribution of codecarboxylase, qualitatively and quantitatively, is the same whether the coenzyme is assayed with lysine apodecarboxylase or tyrosine apodecarboxylase (33).

2. During the preparation of codecarboxylase concentrate from yeast, the degree of purification achieved at each stage in the preparation is the same whether the coenzyme activity is assayed against lysine codecarboxylase or tyrosine codecarboxylase (33).

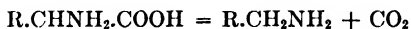
3. Codecarboxylase has the same stabilities and is precipitated under the same conditions by lead, silver, etc., whichever of the apoenzyme preparations is used for assay (33).

4. A boiled preparation of any one of the four enzymes will act as a source of coenzyme for any one of the four apoenzymes.

5. "Pyridoxal phosphate" preparation will act as coenzyme for the four apoenzymes (7).

## IX. Biological Function of Amino Acid Decarboxylases

Bacteria produce amino acid decarboxylases in response to growth in an acid environment. Hanke and Koessler (42) suggested that bacteria produce amines under these conditions as "a protective mechanism resorted to when the accumulation of H ions within the organism's protoplasm is incompatible with its normal life processes." The decarboxylation reaction:



occurring at an acid pH results in the liberation of carbon dioxide into the gaseous phase and a consequent change toward neutrality in the reaction of the aqueous environment. When bacteria with active amino acid decarboxylases grow in an amino acid medium adjusted to an acid pH there is a marked shift of the pH during growth toward neutrality (31) and it may be that the production of the decarboxylases under such conditions is a neutralization mechanism evolved by the cell as a method of stabilizing the internal environment against unfavorable changes in the external



environment. In the opposite condition where growth takes place in an alkaline environment, we find that the formation of amino acid decarboxylases is completely suppressed and that amino acids are attacked, instead, by deamination with liberation of ammonia and a consequent tendency toward an acid reaction (31). The further fact that both decarboxylases and deaminases are produced by bacteria in culture only toward the end of active cell division, would also suggest that these enzymes have no highly important function during active division or the anabolic phase of bacterial existence, but are mechanisms evolved to enable the cell to continue to exist in an environment rendered unfavorable by its metabolism during the early stages of growth.

Carbon dioxide is an essential metabolite for many, probably all, bacteria (29, 35, 66, 67), and it is possible that amino acid decarboxylases have a further function in that they act as a source of carbon dioxide in growth conditions in which the retention of carbon dioxide in the medium must be strictly limited by solubility considerations.

The distribution of codecarboxylase indicates that it must have functions other than those of the coenzyme of the bacterial amino acid decarboxylases. Codecarboxylase is found in bacteria such as *Pseudomonas pyocyanea* which do not produce amino acid decarboxylases and in many other cells, including yeast and animal and plant tissues, in which these enzymes have not as yet been demonstrated. Certain mammalian tissues contain enzymes which decarboxylate histidine, tyrosine, and dihydroxyphenylalanine, but it is uncertain what relation, if any, these enzymes bear to the bacterial amino acid decarboxylases (13, 45, 71) although evidence obtained from a study of the action of inhibitors suggests that the histidine decarboxylase of animal tissues contains a carbonyl group (70). "Pseudo-pyridoxine," or pyridoxal, acts as growth factor for many bacteria, but the formation of amino acid decarboxylases does not seem to be essential for growth *per se* as, for example, *Streptococcus faecalis* can grow in the presence of amounts of pyridoxine insufficient to evoke the synthesis of the coenzyme of tyrosine decarboxylase (10). Thus growth will take place in the absence of tyrosine decarboxylase activity but the author has shown that the total amount of growth that will take place in the presence of fermentable carbohydrate is greater if the tyrosine decarboxylase is active, than if it is not, suggesting again that the function of the decarboxylase is that of a buffering or neutralization mechanism. Snell (62) has found that pyridoxamine is equally effective with pyridoxal as a growth factor, but the former cannot act, whether phosphorylated or not, as codecarboxylase (38). A further function of pyridoxal, and one which may be involved in

its growth factor activity, is indicated by the recent work of Snell (59) who has shown that the interconversion of pyridoxal and pyridoxamine will effect a chemical transamination at high temperatures and that the transaminase activity of pyridoxine-deficient animal tissues is subnormal but can be restored by the addition of pyridoxine, pyridoxal, or pyridoxamine in the presence of adenosine triphosphate (55). If these findings can be applied to bacteria, they would suggest that pyridoxal phosphate will act as codecarboxylase when growth takes place in an acid environment and as cotransaminase when growth occurs in neutral or alkaline environments.

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# **ENZYME PROBLEMS IN RELATION TO CHEMOTHERAPY, "ADAPTATION," MUTATIONS, RESISTANCE, AND IMMUNITY\***

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## I. Introduction

An understanding of the problems indicated by the title of the present study requires their treatment in relation to one another. By emphasizing the biochemical aspects involved, it is hoped that some light may be thrown on the chemical nature of various hitherto undefined biological processes.

Of these problems, "adaptation" represents a concept according to which it has been claimed that cells synthesize certain enzymes as a result of merely being in contact with certain substrates. Since this concept implies that a cell can readily acquire a *building-up* process, it fringes on the processes involving genetic variations. An objective analysis of the experimental data, from which this concept is derived, is necessary, not only because of the theoretical interest involved, but because of the relation of adaptation to chemotherapy, resistance to drugs, immunological variations, etc.

Besides the building-up process implied by the "adaptation" concept, the term has been used also to denote the acquisition of resistance to drugs. The contradiction arising from the use of the same term for two basically different processes—enzyme synthesis stimulated by the action of substrates, and development of resistance to the suppressive action of toxic agents—is very striking.

Brief reference may be made to still another application of the adaptive enzyme concept. According to Burnet (14), the production of antibodies is comparable to the production of adaptive enzymes. At this time, without going into an extensive analysis of the implications of this analogy, it may be pointed out that antigens stimulate the production of modified globulins which function as specific inhibitors of antigen activity. Antigens function as catalysts (154); they are not metabolized but are inhibited

by antibodies. The relationship of antigen to antibody is not, therefore, similar to the adaptive enzyme-substrate relationship. The antibody, once formed, is not known to stimulate the synthesis of a specific enzyme which produces the same antibody in the absence of a specific antigen. For, if this were an established process, the subsequent injection of antibody into a previously immunized animal which had ceased to produce demonstrable antibody would stimulate increased production of identical antibody molecules. Facts do not support this view, nor the analogy between the so-called adaptive enzyme process and antibody production.

Certain investigators have also used the term "bacterial immunity" for resistance to drugs, meaning, presumably, the production by a drug-resistant strain of a substance assumed to neutralize the toxic agent in a manner comparable to the toxin-antitoxin reaction. There is neither an experimental nor a theoretical basis for such an assumption. As will be discussed later, toxic agents, unlike antigens, suppress and eventually completely abolish, and do not stimulate, the synthesis of drug-sensitive cellular factors.

In view of the above considerations, it may, at this time, be pointed out that there is no specific *raison d'être* for the use of the terms "bacterial immunity" and "adaptation." Eliminating the confusion created by these nondefinable terms, we are left with a consideration of the term, "resistance," and the process it represents. On the other hand, the gap created by the elimination of the adaptive enzyme concept is filled by the recognition and emphasis of the building-up processes of cells subsequent to the acquisition of genetic factors from other cells of the same species but of higher function and structure.

## II. Mode of Action of Antibacterial Agents

Since the introduction of sulfonamides as antibacterial chemotherapeutic agents, numerous hypotheses have been formulated to explain their mode of action. Of these, many have failed to give a satisfactory explanation of the mechanism and, therefore, have become principally of historical interest (64a, 160). Within the last four years, certain other highly interesting concepts have been formulated. These pertain principally to the physicochemical properties of drugs. Deserving special consideration are the following:

The theory by Bell and Roblin (6b) deals with the electro-negative, ionic, and acidic characters of the  $-\text{SO}_2-$  group of sulfonamides, and the influence of substituents in the  $-\text{SO}_2-\text{NH}_2$  group in relation to the positive charge on the *p*-amino group in respect to the ability to combine with a protein.

Klotz (80a) applies the law of mass action to drug-protein combinations and relates bacteriostatic action to reversible combinations between the basic form of the drug and the "neutral" form of the protein.

Kumler *et al.* (86a, 86b, 86c) emphasize the relation of resonance to the negative character of the  $-\text{SO}_2-$  group and point out that certain antibacterial compounds are incapable of ionizing as acids; however, see Bell *et al.*, (6a) and Bordwell and Klotz (9a).

According to Johnson *et al.* (74b), sulfanilamide, in neutral solutions, acts on luminescence in a manner very much resembling that of hydrogen ions at certain acidities. Like the hydrogen ion equilibrium, the sulfanilamide equilibrium is said to involve a ratio of approximately one inhibitor molecule to one enzyme molecule. They assume that sulfonamides combine through their amino groups with an ionizable hydrogen of the bacterial enzyme. This blocks the ionization of the enzyme. However, *p*-aminobenzoic acid (PAB) can overcome this effect by ionization of its carboxyl group, which sulfanilamide cannot do. The ability of the drug-enzyme compound to ionize permits bacterial growth, whereas luminescence requires not only ionization, but also the giving up of a particular electron, which, apparently, neither the PAB-enzyme nor the sulfanilamide complex can do.

These studies attempt primarily to locate in the molecule of the drug a specific property to account for its action on bacteria. This property (or properties) has served as a basis for conjecturing the hypothetical nature of the reactive element in bacteria. As indicated above, this is assumed by some to be an *ionizable hydrogen* of certain bacterial proteins, which combines with the basic group of the drug. Another premise, which appears to us to be of questionable validity, is that *p*-aminobenzoic acid is the only substance whose metabolism is interfered with by sulfonamides. Also questionable is the use of the sulfonamide-*p*-aminobenzoic acid antagonism as the basis for the formulation of the above concepts. For it must be remembered that sulfonamides are antagonized by such widely different structures as those possessed by purines, amino acids, riboflavin, cocarboxylase, methylene blue, etc., which may indicate that bacteria do not thrive by means of rigid or straight-jacketed mechanisms, but that they exercise versatile metabolic activities.

It is a plain fact that the first requisite for a drug to show antibacterial action is that it enter into combination with one or more vital groups of the bacterial cell. Whatever may be the exact nature of this combination, the fact remains that the above physicochemical explanations do not give us a hint regarding the chemical nature of the enzymes which play a vital

part in the biology of bacteria and are specifically inhibited by these drugs. Why the other enzyme proteins are not similarly affected, despite the fact that as proteins they must have *ionizable hydrogens*, cannot be answered by these theories. Other more critical factors, *i. e.*, structural and configurational compatibilities, must therefore enter into our considerations. As discussed previously (160, 162), and as will be further discussed in Part III of this study, those chemotherapeutic substances which are structurally compatible with the coenzyme molecule, or at least with part of it, may competitively combine with the protein component of the respiratory enzymes, forming inactive "drug-protein-coenzyme" complexes. This point receives emphasis from Mudd (124a) who has pointed out that, "of the almost five thousand sulfonamides which have been prepared, the few that have proved definitely superior to sulfanilamide have, in each case, a substituent on the N<sup>1</sup>-nitrogen, which is a component of a respiratory coenzyme (pyridine thiazole, pyrimidine)." These facts in mind, the determination of the nature of the enzymes upon which the drugs act would be, therefore, best achieved by a study of the behavior of the drugs on the chemical reactions mediated by bacterial enzymes of primary physiological importance. On the other hand, it would seem that orienting information could also be obtained by studies of the effects of new compounds, synthesized for therapeutic purposes, on the respiratory enzymes in metabolic environments, which take into consideration certain factors which operate *in vivo* (158).

The above physicochemical explanations cannot be reconciled with the fact that sulfonamides inhibit certain specific enzymes but are ineffective toward other specific enzymes present in a given cell. Do these sulfonamide-insensitive enzymes lack *ionizable hydrogens*? Neither do physicochemical explanations explain the changes that a cell may undergo during the development or acquisition of resistance to drugs. Can drug resistance be explained by supposing that the enzyme proteins have lost this "vital" *ionizable hydrogen*, which, on the basis of the above physicochemical formulations, is required for combination with the basic group of the drug? Or, do bacteria which are not sensitive to drugs lack proteins possessing ionizable hydrogens? These are some of the questions one must ask, not as criticisms directed at the above interesting experimental studies and the concepts emanating from them, but that we may be able to approach this highly complex problem in a comprehensive manner.

With these considerations in mind, an attempt will be made to integrate various observations concerning the two principal theories which are now current; these attribute the critical action of sulfonamides to: (a) antago-



nism between sulfonamides and *p*-aminobenzoic acid (44, 208), and (b) inhibition of the respiratory enzymes of bacteria (158, 160, 162). As described on pages 58, 63, and 108), the synthesis and breakdown of amino acids are brought about through the mediation of the oxidative enzymes; *p*-aminobenzoic acid, if it were a normal cell metabolite of bacteria, would require for its synthesis reactions similar to those required for the synthesis of aromatic amino acids. The two theories, therefore, concern the inhibition of enzyme reactions which are fundamentally interrelated, which, indeed, constitute parts of the same basic process. It would, therefore, seem that our primary concern should be the nature of the enzymes which the inhibitors and antagonists act upon.

### 1. Antagonism between Sulfonamides and *p*-Aminobenzoic Acid

***p*-Aminobenzoic Acid as Growth Factor.**—In 1940, Woods (208) reported that the addition of *p*-aminobenzoic acid (PAB) to culture media antagonized the inhibitory action of sulfanilamide toward the growth of bacteria. Fildes (44) proposed that PAB is an essential metabolite normally associated with an enzyme, and that sulfonamides exercise their antibacterial action by interfering with the metabolism of this substance. In subsequent years, researches with PAB have overshadowed all other attempts to discover the mode of action of sulfonamides. Assumptions concerning the role of PAB have received impetus from a few observations with a limited number of saprophytic microorganisms to the effect that the addition of PAB to synthetic media initiates or increases growth. It has, for example, been reported that PAB is a growth factor for *Clostridium acetobutylicum* (144), *Acetobacter suboxidans* (88), a mutant strain of *Neurospora crassa* (189), and *Streptobacterium plantarum* (86). It has also been reported that PAB stimulates the growth of, and the production of lactic acid by, *Lactobacillus arabinosus* (94).

Observations of Lampen and Peterson (87) on the requirement of PAB by *Cl. acetobutylicum* may briefly be described. Testing the efficacy of biotin (0.001  $\mu$ g. per ml.) and PAB (0.05  $\mu$ g. per ml.) individually or in combination as growth factors in a simple salt-glucose medium, two strains of the butyric acid bacteria required biotin but not PAB; the growth of eight strains of *Cl. acetobutylicum* [including strain 862 used by Rubbo and Gillespie (144)] was not supported by either biotin or PAB; in the presence of both factors, appreciable growth was obtained. The growth of two out of eleven strains of *Cl. butylicum* tested required the presence of both biotin and PAB; individually, they were inadequate. The remaining nine strains grew in the presence of biotin but not of PAB. In the presence of both biotin and PAB, the growth of the nine strains was equal to, but not more than, that in biotin alone. These results show that the effect exercised by PAB on the growth of these particular strains is conditioned by the presence of biotin.

A similar interdependence between PAB and thiamin for the promotion of the growth of a pink yeast, *Rhodotorula aurantica* (Saito) Lodder, is reported (140). This yeast was isolated from an aged beer yeast culture by M. Wahl. With thiamin or PAB alone, the pink yeast failed to grow. The intensity of the pink color was related to the supply of PAB. In media with less than an optimum quantity of PAB, the color was paler and tended toward orange as compared to the deeper pink which developed when more PAB was supplied. In the presence of 10  $m\mu$  moles of thiamin and 0.1 to 1  $m\mu$  mole of PAB, growth was optimal. Growth was less with 10  $m\mu$  moles of PAB than with 1  $m\mu$  mole. An inhibitory effect therefore was operating at higher concentrations of PAB, as was observed with *Cl. acetobutylicum*.

In contrast, PAB has been shown to exercise no growth-promoting effects on pathogenic bacteria. In fact, as will be discussed more fully later, a great number of reports show that PAB actually inhibits the growth of many bacteria, molds, enzymes, and viruses. In these inhibitions, as well as in its antagonistic behavior to sulfonamides, PAB behaves like a non-specific sulfonamide-like inhibitor.

The idea that sulfonamides inhibit bacteria by interfering with their metabolism of PAB derives its principal support from the observation that PAB is a growth factor for a limited number of saprophytic microorganisms, and also from the results of experiments with systems to which PAB has been *added from the outside* and found to negate the inhibitory action of sulfonamides. The results obtained from such experiments cannot, however, be extended or applied to metabolic processes carried out by bacteria under normal physiological conditions. As will be discussed below, there is as yet no convincing chemical or physiological evidence regarding the metabolism or synthesis of PAB by bacteria in general. PAB has been isolated from yeast but not from bacteria or their culture fluids.

In the absence, therefore, of rigid experimental evidence that PAB is synthesized by pathogenic bacteria under normal conditions, investigation must be made into the following: (a) the mechanism of the antagonism between sulfonamides and PAB added to bacterial system; and (b) the action of sulfonamides on bacterial respiratory enzymes from the point of view of a more comprehensive interpretation of the mode of action of chemotherapeutic agents—an interpretation which takes into account the fundamental processes of cellular physiology.

**Absence of *p*-Aminobenzoic Acid as a Stoichiometric Component of Proteins.**—The position of PAB as a biological substance is uncertain. At present, it is very seldom spoken of as a vitamin factor.

Miller *et al.* (122) analyzed several highly purified proteins before and after acid and alkali hydrolysis for their PAB content. These proteins were crystalline beef liver catalase, concanavaline A, renin, urease, two phosphorylase proteins (one with and the

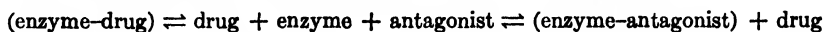
other without adenylic acid), yeast polypeptidase (electrophoretically homogeneous), and two samples of rabbit muscle extract. Using the whole proteins or their hydrolyzates in microbiological tests, results were obtained which seemed to indicate the presence of PAB in the materials analyzed. The amounts of PAB estimated were, however, far less than the amount required to satisfy the stoichiometric relationship of one molecule of PAB to one molecule of protein. For example, it was found that crystalline phosphorylase (with a probable molecular weight of 340,000 to 400,000) and catalase (molecular weight, 250,000) would have to have molecular weights of 10,500,000 and 7,200,000, respectively, in order to satisfy the one-to-one molecular ratio. The same discrepancy was observed with the other proteins. These observations demonstrated that PAB is not a chemically bound component of homomolecular proteins, as would be the case if PAB were a component of a conjugated protein functioning as a coenzyme or vitamin or an amino acid. Miller *et al.* concluded that the substance, claimed to be PAB, was a contaminant of the proteins studied.

**Nonutilization of *p*-Aminobenzoic Acid in Animal System.**—Lustig *et al.* (105) placed mice on a preliminary diet for eight to twelve weeks, during which period the mice developed achromotrichia. Twenty-four hours after treatment with *p*-aminobenzoic acid labeled with N<sup>15</sup> the mice were exsanguinated and the skin, heart, lung, liver, kidney, gastrointestinal tract, spleen, recovered blood, and urinogenital organs analyzed by the mass spectrometric method for PAB. They failed to find the storage or utilization of N<sup>15</sup>. These results show that PAB is not taken up for the synthesis of the components of the tissues analyzed.

***p*-Aminobenzoic Acid in Yeast as Component of an Unusual Polypeptide Chain.**—The presence of PAB in the yeast cell has been reported (7, 144). Ratner *et al.* (138) reported that PAB is a component of a polypeptide chain made exclusively of glutamic acid; 400 mg. of this polypeptide, containing 8.0% PAB, was isolated from 50 kg. of dried yeast, giving one part of PAB in 1,562,500 parts of yeast. On the basis of the evidence available, the structure of the compound was formulated as a chain of ten to twelve glutamic acid residues, the chain being linked to the carboxyl group of one PAB molecule. In this polypeptide chain, the amino group was diazotizable and therefore free; nevertheless the polypeptide did not exercise antisulfonamide properties.

## 2. Competition among Drugs; Drugs and Antagonists

It is a recognized fact that substances possessing physicochemical properties comparable to inhibitors of enzyme systems generally antagonize the physiological effects of these inhibitors or drugs. This antagonism finds its explanation in the fact that both the drugs and their antagonists combine with the same site or active group of the enzyme, and that only drug-enzyme combinations are physiologically or pharmacologically effective. In contrast, antagonist-enzyme combinations are physiologically ineffective, except in preventing the drug from combining with the enzyme. This relationship can be schematized in the following manner:



The above relationships may be illustrated by the following observations. It has been found that ephedrine is capable of antagonizing various actions

of adrenaline (8, 135). This antagonism is assumed to indicate that ephedrine combines with a particular enzyme system, producing a physiologically ineffective complex, thereby preventing the action of adrenaline on the same system. A more striking example is the antagonism exercised by the higher members of the homologous series of cations  $R \cdot N^+(CH_3)_3$  ( $R = C_7H_{15}$  to  $C_{16}H_{33}$ ) to the muscarine-like action of the lower members ( $R = CH_3$  to  $C_6H_{11}$ ) on the frog's muscle. The lower members are reported to act additively with acetylcholine and to be antagonized by atropine.\* These effects are explained by assuming that all the  $R \cdot N^+(CH_3)_3$  cations combine with the receptors for acetylcholine but that the combination is physiologically ineffective for the higher members (139).

Similarly, a strong antagonism between N-allylnormorphine and morphine has been reported by various investigators. According to Unna (195), N-allylnormorphine is about as toxic as morphine, but much less effective in raising the threshold for pain in mice. In contrast to morphine, small doses of N-allylnormorphine do not depress respiration; however, it prevents or abolishes the actions of morphine, such as analgesia in mice, respiratory depression in rabbits, and toxic manifestations in cats and dogs; it reduces mortality from morphine in mice from 28 to 88%. Apparently, then, N-allylnormorphine, by virtue of its chemical relationship to morphine, exerts its action upon the same centers as morphine, rendering them less sensitive to the latter. See also Hart *et al.* (143).

In certain isolated enzyme systems, there is another type of antagonism which prevents the accumulation of toxic reaction products. According to Quastel (135), the addition of benzedrine at low concentrations to brain cortex respiring in glucose medium neither increases nor decreases the respiration, nor does it neutralize the diminution of respiration due to the presence of narcotics. In the presence of amines, such as tyramine or isoamylamine, the respiration of brain *in vitro* undergoes marked diminution. The addition of benzedrine, however, to such a system neutralizes the inhibiting action of these amines. The inhibition of the respiration of brain is due mainly to the toxic action of the aldehyde produced as the oxidation product of tyramine, etc. Benzedrine, resistant to oxidation, owes its stimulating action to its ability to successfully compete with amines for the amine oxidase of brain, thereby reducing the rate of formation of

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\* *In vitro* tests have shown that ether and chloroform interfere with combination between rabbit serum and pilocarpine (184b); peptone and sodium citrate have been shown to interfere with combination between atropine and rabbit serum, and atropine sulfate with combination between rabbit serum and pilocarpine (184c). Since atropine *per se* strongly combines with rabbit serum, there is no doubt that atropine displaces pilocarpine from combination with rabbit serum.

the toxic aldehyde. In relation to the discussions which will follow, it must be noted that benzedrine is a mild inhibitor of amine oxidase; and it is as an inhibitor that it exercises its affinity for the enzyme and thus its antagonism to tyramine and a long list of other amines studied. On the basis of these investigations, it is suggested that the action of benzedrine *in vivo* is linked with its ability to compete with amines which give rise by oxidation to toxic substances. It may be suggested at this point that these observations be kept in mind in analyzing the facts regarding the relationship of the inhibition by sulfonamides of bacterial respiratory enzymes to the chemotherapeutic action of these drugs *in vivo*.

Kensler *et al.* (76, 78) reported that *p*-phenylenediamine and several of its methyl derivatives strongly inhibited fermentation in a washed yeast system in which diphosphopyridine nucleotide was the limiting factor. This inhibition could be prevented by the use of higher coenzyme concentrations, indicating a successful competition by the inhibitor with the coenzyme for an enzyme protein which requires the coenzyme to complete its catalytic function.

Similarly, in experiments with the carboxylase-coccarboxylase system, at a cocarboxylase concentration of  $1.56 \times 10^{-5} M$  (15  $\mu g.$  per 2 ml.), *N,N*-dimethyl-*p*-phenylenediamine ( $5 \times 10^{-4} M$ ) completely inhibited activity. At a  $4.0 \times 10^{-4} M$  (200  $\mu g.$  per ml.) concentration of cocarboxylase, inhibition was absent. At this cocarboxylase concentration the molar inhibitor/cocarboxylase ratio was 1.2.

The addition of large amounts of diphosphopyridine nucleotide to the carboxylase-cocarboxylase system did not prevent the inhibition of this system by the above type of inhibitor. Conversely, the addition of large amounts of cocarboxylase to the fermenting system in which diphosphopyridine nucleotide was the limiting factor did not prevent the inhibition in that system.

Related to the above observations are the results of a study by Sevag *et al.* (158, 163). It was observed that the inhibition of bacterial and yeast carboxylases by sulfathiazole was abolished by both cocarboxylase and *p*-aminobenzoic acid. The antagonistic molar ratios of sulfathiazole/cocarboxylase were from 200 to 600, and those of sulfathiazole/*p*-aminobenzoic were from 1 to 20. The reversal by cocarboxylase of the inhibition is in harmony with the highly specific activities of the limiting factors in counteracting the inhibitory action of dimethyl-*p*-phenylenediamine compounds. Since *p*-aminobenzoic acid is not a limiting factor for the carboxylase system, its ability to counteract the inhibitory action of sulfonamides is of nonspecific nature.

It has been known for many years that malonate\* specifically inhibits bacterial and tissue succinoxidase. This was thought to be due to the similarity in structure between malonate and succinate. Very recently, however, Vennesland and Evans (199) reported that the oxidation by tissue of oxalacetic acid, a derivative of succinate, yielded malonate. Malonate, therefore, inhibits the very enzyme which produces it, in a manner similar to the inhibition of amine oxidase by aldehyde, as previously discussed. It is significant that the inactivation of succinoxidase by oxidized glutathione is counteracted by malonate (70). Thus, malonate, which itself inhibits the enzyme, protects it against oxidized glutathione. Malonate has also been reported to protect succinoxidase against the toxic effects of compounds having the quinoid structure (134). At 50% inhibition, the molecular ratio of succinate to malonate was 25 to 1. Since the dissociation constant for the succinoxidase-malonate system was found to be only one-fiftieth that of succinoxidase-succinate, the failure of quinone to inhibit the enzyme in the presence of malonate was probably due to the fact that there were virtually no uncombined enzyme molecules for quinone to attack.

**Competition between Inhibitors and Antagonists.**—The observations discussed above have been presented to show that these antagonisms represent a common biological occurrence, and are, therefore, of value in analyzing and evaluating the results of studies with antibacterial agents and their antagonists. The significant point to be noted is that antagonists exercise their effect by completely or partially restoring the normal functions of physiological systems susceptible to inhibitors or drugs. This restoration, in the case of bacterial growth, may be the result of stimulation by the antagonist acting as a growth factor, or it may simply be the result of a physicochemical action on the bacterial enzymes. The antagonism of *p*-aminobenzoic acid to sulfonamides may or may not indicate a "co-function" of a PAB-specific enzyme system. There are, however, as yet no experimental data to show that either sulfonamides or PAB undergo a reversible chemical change, as certain coenzymes and amino acids do. Such change might account for the behavior of sulfonamides and of PAB toward bacteria. In the absence of such specific information, antagonism between sulfonamides and PAB may perhaps best be viewed as a physicochemical reaction.

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\* That malonic acid is a biological metabolite has been demonstrated by Raistrick (136b). It is a component of, and is liberated by alkaline hydrolysis of, a high molecular weight polysaccharide. The other sole product of hydrolysis is glucose. This polysaccharide was produced from glucose by a strain of *Penicillium luteum*.

Mathematical analysis of experimental data by Wyss (212), Strauss *et al.* (185a), and Gaddum (48a) has shown that sulfanilamide (SA) and PAB probably compete for the same site in various bacteria. When PAB wins, the sulfonamide loses its effect as a therapeutic agent. PAB was found not to exercise any growth-stimulating effect. Maximum growth velocities were identical with or without PAB. Wyss states that if a concentration of 0.005 mg. per 100 ml. of PAB were present, either as an impurity or as a product of bacterial synthesis, in addition to the added amount (0.01 mg. per 100 ml.), it could be detected in the system. However, such was not the case. In this study the proportionality between the molecular concentrations of SA and PAB was 2000:1. Under these conditions the complete inhibition of the growth of *Escherichia coli* by 2000 molecules of SA was reduced one-half by one molecule of PAB. This indicates that PAB exercised a greater affinity than SA for a bacterial enzyme system.

### 3. Interpretation of Molar Ratios of Inhibitor/Antagonist

One molecule of PAB has been found to completely antagonize the inhibition of bacterial growth by 5000 to 23,000 molecules of SA. PAB in these experiments, as in those cited above, did not accelerate, initiate, or increase the growth of the bacteria studied (185a, 208, 212). This tremendous ratio (SA/PAB) has induced the postulate that the antibacterial action of SA is due to its competitive inhibition of enzymes which normally interact with PAB or with a closely related substance.

McIlwain (109) has stated: "It is difficult to picture the mode of action of so small a proportion of PAB except by a specific enzyme mechanism." In rejecting the "narcotic" hypothesis offered by Johnson (74) in explaining the antagonism of urethan to sulfanilamide, he stated further that (a) while one molecule of PAB is required to antagonize 5000 molecules of SA, from one to 100 molecules of urethan are required to bring about the same effect; (b) the ratios between the concentrations of SA to PAB are constant and indicate a competitive reaction, but those between SA and other antagonists are not; and (c) because of the structural similarity, a mutual specificity controls the antagonism of PAB, nicotinamide, and pantothenate, respectively, to sulfanilamide, pyridine-3-sulfonamide, and pantoyletaurine.

But the magnitude and assumed constancy of ratios between an inhibitor and its antagonists are not valid bases for a postulate regarding the nature of the effect of the antagonist. The results presented in Table I seem to favor the opposite view, *viz.*, that the *said tremendous ratio does not exist in a system in which PAB functions as a necessary growth factor*; for example, a SA/PAB ratio of 150 has been determined for *Streptobacterium plantarum*. In systems where the growth of *Lactobacillus arabinosus* and *L. pentosus* was increased several fold in the presence of PAB, the ratio of SA/PAB was likewise low (5 to 150). In contrast (with the doubtful exception of *Clostridium acetobutylicum*, as the results of Lampen and Peterson seem to show), in all other cases where PAB does not exercise any growth-stimulating effect, the antagonism ratio, SA/PAB, ranges on the

average from 5000 to 25,000. This relationship seems to hold for other antagonistic systems, such as the systems studied by Wo White, Snell and Mitchell, Kuhn *et al.*, etc., as shown in Table I.

It is also of importance to compare the antagonism ratios of PAB to the various sulfonamides. Table I (Brueckner) shows that: SA/PAB = 21,000, SP/PAB = 750, SD/PAB = 200, and ST/PAB = 24\* (see also results by Kuhn *et al.* in the table). These and similar observations emphasize the fact that the degree of physicochemical affinities is a determinant factor in conditioning the magnitude of antagonism ratios or the inhibition index (Woolley and White). In addition to the influence of physicochemical affinities, the presence of nonbacterial proteins, metabolites, vitamins, and amino acids added to the system and synthesized during growth, are factors which exert significant effects on the magnitude of the ratios.

From the tabulated observations, it may be concluded that *high ratios of molecular concentrations of inhibitors to antagonists are, in general, related to antagonists which do not exercise growth-promoting effects while counteracting*

\* Why the sulfonamide/PAB ratios for SA, SP, ST, and SD vary widely has, no doubt, been asked. Mudd (124a) made the suggestion that this may be due to the possibility that SA and the N<sup>1</sup>-substituted group (which structurally resembles the coenzyme or part of it, and competes with the coenzyme for the reaction sites, on the enzyme protein) are capable of combining, at two reaction sites, in appropriate steric relationship, on the enzyme protein. In this connection, the results of our study (162) may be of some interest. The comparative inhibitory effects of sulfonamides containing the pyrimidine, pyridine, and thiazole rings on isolated carboxylase systems of various organisms were determined. The thiazole group exercised a high degree of specific inhibition on the isolated carboxylase systems of *S. aureus* and *E. coli*. On the other hand, the carboxylase system of yeast was equally affected by all the sulfonamides containing pyrimidine, pyridine, and thiazole rings; sulfamethyldiazine was, however, completely ineffective on the systems studied. These observations indicated that weakly active sulfanilamide was potentiated by the introduction of the above-mentioned rings. However, pneumococcal cells lack carboxylase; thus the antipneumococcal activity of sulfathiazole made it clear that this drug also acts nonspecifically on systems not containing the thiazole ring.

Klotz (80a) accounts for the differences in the ratios of sulfonamide to PAB from drug to drug and from pH to pH by calculations based on the law of mass action involving reversible combinations between the basic form of the drug and the protein. He relates the drug potency to the acid ionization constant of the sulfonamide and the pH of the solution. Translating the calculations of Klotz into plain numerical values, we find that the dissociation constant of the enzyme-sulfonamide complex for sulfanilamide is 1000-fold greater than for sulfathiazole, and 100-fold greater than for sulfapyridine. A comparison of the sulfonamide/PAB ratios (Brueckner) of SA and ST, and SA and SP shows, respectively, a 1000- and 30-fold difference.

As we see it, if the degree of drug activity can be related to the degree of the basicity of the *p*-amino group (which combines with the enzyme proteins), then the substituent groups in the N<sup>1</sup>-position ( $-\text{SO}_2\text{NH}_2$ ) decreases the acidity of the latter group, causing an increase in the basicity of the *p*-amino group. Thus, the *p*-amino group in ST will manifest a greater basic property than the corresponding group in SA. A combination between ST and protein, being comparable to a salt of a relatively strong base and a weak acid, will undergo dissociation to a lesser degree than the corresponding complex with SA. As a result, a greater amount of PAB will be required to displace ST from its combination than would be required to displace SA.



TABLE I  
RATIOS OF INHIBITORS TO ANTAGONISTS IN RELATION TO PRESENCE OR ABSENCE OF GROWTH-PROMOTING EFFECTS EXERCISED BY THE ANTAGONISTS

Microorganisms	Inhibitor	Antagonist		Inhibitor-Antagonist	Reference
		Name	Effect on growth		
<i>S. pyogenes</i>	Sulfanilamide (SA)	p-Aminobenzoic acid	None	5,000-25,000	Woods (208)
<i>S. pyogenes</i>	Sulfanilamide	p-Aminobenzoic acid	None	5,000	McIlwain (109)
<i>E. coli</i>	Sulfanilamide	p-Aminobenzoic acid	None	5,000-25,000	Woods (208)
<i>E. coli</i>	Sulfanilamide	p-Aminobenzoic acid	None	2,000*	Wys et al. (212a)
<i>E. coli</i>	Sulfanilamide	p-Aminobenzoic acid	None	1,870,000	Strauss et al. (185a)
<i>S. aureus</i>	Sulfanilamide	p-Aminobenzoic acid	None	21,000	Brueckner (12)
<i>S. aureus</i>	Sulfanilamide	p-Aminobenzoic acid	None	4,660*	Wys et al. (212a)
<i>L. casei</i>	Sulfanilamide	p-Aminobenzoic acid	None	8,000	Snell and Mitchell (169)
<i>S. planitum</i>	Sulfanilamide	p-Aminobenzoic acid	Growth factor	150*	Kuhn et al. (85)
<i>L. arabinosus</i>	Sulfanilamide	p-Aminobenzoic acid	4- to 8-fold increase	5	Snell and Mitchell (169)
<i>L. pentosus</i>	Sulfanilamide	p-Aminobenzoic acid	4- to 8-fold increase	5	Snell and Mitchell (169)
<i>Cl. acetobutylicum</i>	Sulfanilamide	p-Aminobenzoic acid	Growth factor (?)	26,000	Rubbo and Gillespie (144)
<i>L. casei</i>	Sulfanilamide	Guanine	None	1,200	Snell and Mitchell (169)
<i>L. casei</i>	Sulfanilamide	Xanthine	None	880	Snell and Mitchell (169)
<i>L. casei</i>	Sulfanilamide	Hypoxanthine	None	800	Snell and Mitchell (169)
<i>L. casei</i>	Sulfanilamide	Adenine sulfate	None	1,250*	Snell and Mitchell (169)
<i>L. pentosus</i>	Sulfanilamide	Adenine sulfate	7-fold increase	<1	Snell and Mitchell (169)
<i>S. lactis R</i>	Pyrimidin	Pyrimidine and thiazole	None	5,000,000*	Woolley and White (210)
<i>S. pyogenes</i> H69D	Pyrimidin	Pyrimidine and thiazole	None	4,000,000*	Woolley and White (210)
<i>E. coli</i>	Pyrimidin	Pyrimidine and thiazole	None	2,000,000*	Woolley and White (210)
<i>L. arabinosus</i>	Pyrimidin	Pyrimidine and thiazole	None	40,000*	Woolley and White (210)
<i>L. casei</i>	Pyrimidin	Pyrimidine and thiazole	None	5,000,000*	Woolley and White (210)
<i>L. masenteroides</i>	Pyrimidin	Pyrimidine and thiazole	None	5,000,000*	Woolley and White (210)
<i>L. penicillatus</i>	Pyrimidin	Pyrimidine and thiazole	None	5,000,000*	Woolley and White (210)
<i>S. aureus</i>	Pyrimidin	Pyrimidine and thiazole	None	2,000*	Woolley and White (210)
<i>Phlophora cinnamomi</i>	Pyrimidin	Thiamin	Growth factor	12*	Woolley and White (210)
<i>Endomycetes versalis</i>	Pyrimidin	Pyrimidine	Growth factor	130*	Woolley and White (210)
<i>Mucor ramannianus</i>	Pyrimidin	Thiazole	Growth factor	800*	Woolley and White (210)
<i>L. fermentum</i>	Pyrimidin	Thiamin	Growth factor	50*	Sarrett and Cheldelin (146)
<i>S. aureus</i>	Sulfapyridine (SP)	p-Aminobenzoic acid	None	10*	Sarrett and Cheldelin (146)
<i>S. aureus</i>	Sulfapyridine (SP)	p-Aminobenzoic acid	None	21,000	Brueckner (12)
<i>S. aureus</i>	Sulfadiazine (SD)	p-Aminobenzoic acid	None	750	Brueckner (12)
<i>S. aureus</i>	Sulfadiazine (SD)	p-Aminobenzoic acid	None	200	Brueckner (12)

TABLE I (Continued)

Microorganisms	Inhibitor	Antagonist		Inhibitor/Antagonist	Reference
		Name	Effect on growth		
<i>S. aureus</i>	Sulfathiazole (ST)	p-Aminobenzoic acid	None	24	Brueckner (12)
<i>S. plantarum</i>	Sulfanilamide	p-Aminobenzoic acid	Growth factor	150*	Kuhn <i>et al.</i> (85)
<i>S. plantarum</i>	Sulfapyridine	p-Aminobenzoic acid	Growth factor	63*	Kuhn <i>et al.</i> (85)
<i>S. plantarum</i>	Sulfathiazole	p-Aminobenzoic acid	Growth factor	35*	Kuhn <i>et al.</i> (85)
<i>S. plantarum</i>	Sulfanilic acid	p-Aminobenzoic acid	Growth factor	5,000*	Kuhn <i>et al.</i> (85)
<i>S. plantarum</i>	p-Aminobenzopyridine	p-Aminobenzoic acid	Growth factor	48,000*	Kuhn <i>et al.</i> (85)
<i>S. pyogenes</i>	Pantoysulfonamide	$\beta$ -Alanine + $\alpha$ , $\gamma$ -dihydroxybutyric acid	Growth factor	2,000	Ivánovics (72a)
<i>S. pyogenes</i>	Homopantoylsulfonic acid	Hydroxypantothenic acid	Artificial growth factor	20,000	Ivánovics (72a)
<i>S. pyogenes</i>	Pantoylsulfonic acid	Pantothenic acid	Growth factor	500	Ivánovics (72a)
<i>S. pyogenes</i>	Pantoyltaurine	Pantothenic acid	Growth factor	100	Ivánovics (72a)
<i>C. diptheriae</i>	Pantoyltaurine	Pantothenic acid	Growth factor	100	McIlwain (109)
<i>Diplococcus pneumoniae</i>	Pantoyltaurine	Pantothenic acid	Growth factor	100	McIlwain (109)
<i>S. aureus</i>	Sulfathiazole	Tryptophan	Growth factor	17	Sevag and Green (155)
<i>S. aureus</i>	Sulfathiazole	Pantothenic acid	Growth factor	293	Sevag and Green (155)
<i>S. aureus</i>	Sulfathiazole	Riboflavin	Growth factor	622	Sevag and Green (155)
<i>S. aureus</i>	Pyridine-3-sulfonamide	Nicotinamide	Growth factor	1,000	McIlwain (109)
<i>L. arabinosus</i> 17-5	Sulfapyridine	Nicotinic acid	Growth factor	100	Tepley <i>et al.</i> (193)
<i>L. arabinosus</i> 17-5	Sulfapyridine	p-Aminobenzoic acid	4- to 8-fold increase	500	Tepley <i>et al.</i> (193)
<i>E. coli</i>	Aranilic acid (atoxyl)	p-Aminobenzoic acid	None	10,000	Peters (132)
<i>E. coli</i>	Sulfanilamide	Methionine	None	100	Peters (132)
<i>E. coli</i>	Aranilic acid	Methionine	Not apparent	10,000	Peters (132)
<i>E. coli</i>	Sulfanilamide	Methionine	Not apparent	100	Peters (132)
<i>E. coli</i>	Atabrine	Alloxazine-monomonucleotide	Coenzyme	500	Haas (55)
Cytochrome reductase					
Carboxylases:					
Whole yeast	Sulfathiazole	Coccarboxylase	Coenzyme	8,088-53,400	Sevag <i>et al.</i> (157, 163)
Alkaline washed yeast	Sulfathiazole	Coccarboxylase	Coenzyme	322-646	Sevag <i>et al.</i> (157, 163)
Whole <i>Staphylococcus</i>	Sulfathiazole	Coccarboxylase	Coenzyme	215	Sevag <i>et al.</i> (157, 163)
Whole <i>Staphylococcus</i>	Sulfathiazole	p-Aminobenzoic acid	None	10	Sevag <i>et al.</i> (157, 163)
Whole <i>E. coli</i>	Sulfathiazole	p-Aminobenzoic acid	None	20	Sevag <i>et al.</i> (157, 163)

\* Reduction of the 100% to 50% inhibition. The ratios obtained by Woolley and White represent the inhibition index (weight of inhibitor/weight of antagonist).

*the action of inhibitors of bacterial growth.* In contrast, *low ratios appear to be related, on the whole, to antagonists which do function as growth factors.* Since, in almost all systems where pathogenic bacteria were used, the SA/PAB ratios have generally been comparatively high, it seems reasonable to suggest that in these instances the antagonism on the part of PAB does not involve a growth-promoting or metabolite function.

The above conclusion may serve as a useful working hypothesis in understanding the role of various factors involved in antibacterial chemotherapy. For, in those instances where high inhibitor/antagonist ratios are observed, the single role of the antagonist is to displace the inhibitor from the bacterial enzyme system; therefore, depending on the relative degree of affinity for the bacterial enzyme system shown by antagonist and inhibitor, the amount of antagonist required is less than if it were functioning as a growth factor. On the other hand, *when inhibitor/antagonist ratios are low, a double role is being played by the antagonist and therefore a much greater amount of it is required:* (a) *an amount to displace the inhibitor from the critical site of the enzyme system, and (b) an additional amount to fill the growth requirements of the organism after it has been freed from the inhibitor.* In the instances of high ratios, the normal synthesis of growth factors is either less susceptible to interference by the inhibitor, or this interference is readily overcome, requiring very minute amounts of antagonists, as indicated in the table.\* In the instances of low ratios, it is the system involved in the utilization of the growth factor (which it cannot synthesize) that seems to be highly susceptible to the inhibitor, thus requiring a disproportionately greater amount of antagonist for displacing the inhibitor than would be expected from the high antagonism ratios characteristic of systems where the antagonist does not function as a growth factor.

For example, in the work reported by Teply *et al.* 0.05  $\mu$ g. of nicotinic acid per ml. of medium was required for the growth of *Lactobacillus arabinosus*, and 100  $\mu$ g. of nicotinic acid per ml. for completely counteracting the inhibition of growth by 2  $\mu$ g. of sulfapyridine per ml. of medium. This indicates that 99.95% of the nicotinic acid was involved solely in counteracting sulfapyridine. In experiments by Snell and Mitchell, the maximal increase in the growth of *L. arabinosus* was obtained with 0.03  $\mu$ g. of *p*-amino-benzoic acid per ml., but it required 0.3  $\mu$ g. of PAB per ml. to counteract the inhibition by 2.0  $\mu$ g. of sulfanilamide per ml. That is, 90% of PAB was involved solely in the counteraction of SA. Or, in absolute terms, the SA/PAB ratio was 6. In contrast, PAB did not exercise any growth-stimulating effect under similar conditions on *L. casei*, and the SA/

\* This relationship may also explain the absence of inhibition by sulfonamides and penicillin of the growth of gram-negative organisms which possess a high degree of synthetic ability. They are able to synthesize amino acids and vitamins during growth in a medium containing inorganic salts, glucose, and ammonium ion. They may thereby be resistant to these drugs.

PAB ratio was 8000. Again, 1  $\mu\text{g.}$  per ml. of adenine sulfate did not exercise any measurable growth effect on *L. casei*; and, at a ratio of SA/adenine sulfate of 1250, the inhibition was reduced from 90 to 40%. On the other hand, the growth of *L. pentosus* was promoted in the presence of adenine sulfate, and the SA/adenine sulfate ratio was 0.16. Of the 3.0  $\mu\text{g.}$  of adenine sulfate per ml. used, only 0.03  $\mu\text{g.}$  per ml. was required for the growth of *L. pentosus*. That is, 99% of the antagonist was involved solely in the counteraction of 2.0  $\mu\text{g.}$  of SA per ml. In these experiments, PAB and adenine sulfate appear to behave alike, which offers reasonable support to the above conclusions. These conclusions seem to be strongly supported by the results also of Woolley and White (see table). Where the antagonist (thiamin or its components) functioned as growth factor, the inhibition index (weight of pyrithiamin/weight of thiamin) ranged from 10 to 2000. In contrast, where the antagonist was not required as a growth factor, the inhibition index ranged from 40,000 to 5,000,000.

In regard to the significance of inhibitor/antagonist ratios where PAB is definitely involved as a growth factor, the data of Tatum and Beadle (189) are of particular interest. They found that sulfanilamide completely inhibited the growth of a normal strain of *Neurospora crassa* capable of growing in the absence of preformed PAB, as well as that of a mutant strain requiring preformed PAB. In both cases PAB was capable of overcoming the inhibitory action of SA.

Analyzing the data reported by Tatum and Beadle, we see that concentrations ranging from 4 to 250  $\mu\text{g.}$  of SA per 15 ml. of medium did not inhibit the utilization of 1  $\mu\text{g.}$  of PAB by the mutant strain. With concentrations ranging from 500 to 15,000  $\mu\text{g.}$  of SA per 15 ml. of medium, the growth rate gradually declined and complete inhibition occurred with the indicated highest concentration of SA. At a concentration slightly higher than 250  $\mu\text{g.}$  of SA, the growth rate curve began to decline; this therefore represented the critical concentration. Since 0.15  $\mu\text{g.}$  of PAB per 15 ml. is the optimal requirement for growth by the mutant strain, of the 1  $\mu\text{g.}$  of PAB present in the medium 85% was involved in counteracting from 4 to 250  $\mu\text{g.}$  of SA and maintaining the growth rate on a straight line. Since the amount of PAB synthesized by the normal strain was not in excess of that required by the mutant strain, the conclusion by these investigators that antisulfonamide activity of PAB for the mutant strain is  $1/100$  of its activity for the normal strain supports our above conclusion.

Doermann (32) found that in a mutant strain of *Neurospora crassa* the failure to synthesize a growth factor was associated with a specific sensitivity to other compounds. For example, the utilization of preformed lysine by a lysine-less strain was specially interfered with by arginine. A molar ratio, arginine/lysine, of 1 reduced the growth to 50% of that occurring in the arginine-free control culture. At a ratio of 2, growth was completely inhibited. In contrast, the utilization of the endogenous lysine by the wild type of *Neurospora* was not interfered with by high arginine concentrations. Similarly, the utilization of preformed riboflavin by a mutant strain was very strongly inhibited by lumichrome, and somewhat by lumi-

flavin, neither of which inhibited the growth of other strains which were capable of synthesizing their own riboflavin (unpublished observations by Houlahan and Mitchell, cited by Tatum and Beadle, 191).

Snell (168), using pantothenate as growth factor and its sulfonic acid analogue as inhibitor, reported certain quantitative data from which we have calculated the following inhibitor/antagonist molar ratios: For *Lactobacillus arabinosus* (17-5), 173; for *Lactobacillus pentosus* (124-2), 5760; for *Streptococcus lactis* R, 576; for *Propionibacterium pentosaceum* (p-11), 8650; for *Leuconostoc mesenteroides* (p-60), 1730; and for *Saccharomyces cerevisiae*, 5760. These ratios show varying degrees of susceptibilities to the inhibitor by organisms requiring pantothenate for growth. However, using  $\beta$ -alanine instead of pantothenate for the growth of the yeast, the ratio was 576. That is, the synthesis of pantothenate from  $\beta$ -alanine was 10-fold less sensitive to the inhibitor than when pantothenate was used as growth factor. In this respect the following observations are of more definite nature. The growth of *Escherichia coli* was not inhibited in the presence of  $1.8 \times 10^{-2} M$  of inhibitor. In the presence of  $3.6 \times 10^{-4} M$  of inhibitor, the growths of *Staphylococcus aureus*, *Shigella paradysenteriae*, and *Brucella abortus* likewise were not inhibited. These latter organisms did not require pantothenic acid for growth. In agreement with our conclusion, it is seen that those organisms which are capable of synthesizing pantothenic acid during growth are insensitive, while those incapable of synthesizing it are sensitive to the action of pantoyltaurine.

Snell and Shive (170), using *Leuconostoc mesenteroides* p-60, determined the antagonism ratios of pantothenate, which was required for growth, to the following inhibitor analogues: *dl*-N-pantoylethanolamine, *dl*-N-pantoylethylamine, *dl*-N-pantoyl-N-propylamine, *dl*-N-pantoylallylamine and *dl*-N-pantoylglycine. Inhibitor/antagonist molar ratios ranged from 0.06 to 0.14. At these ratios, the inhibition of growth was completely abolished by pantothenate.

McIlwain's (110) results likewise show that *Staphylococcus aureus* and *Eberthella typhosa*, which are capable of synthesizing  $\alpha$ -aminocarboxylic acids, were insensitive to the inhibitory action of  $\alpha$ -aminosulfonic acids. Similarly, *Corynebacterium diphtheriae*, which is capable of synthesizing pantothenic acid from  $\beta$ -alanine, was resistant to pantoyltaurine, and the strain which was exacting with respect to pantothenate was highly sensitive to this inhibitor.

In the light of all the above discussed observations, one may conclude that *high molar ratios of SA/PAB, observed in experiments with pathogenic bacteria, indicate that: (a) PAB does not function as growth factor in these antagonistic reactions; (b) the interference, if any, with the synthesis of PAB is readily antagonized and, therefore, does not suggest being of a consequential nature; and (c) the effective inhibitory action of SA would thus appear to in-*

*volve an interference with the utilization of other growth factors which the organisms are unable to synthesize, and with the respiratory mechanism (Part III).*

#### 4. *Nonspecific Action of p-Aminobenzoic Acid on Isolated Systems*

In the majority of cases, PAB appears to function as a mere antagonist to sulfonamides in a manner comparable to the nonspecific antagonism to sulfonamides shown by urethane, adenine, guanine, xanthine, hypoxanthine, as described above, and by certain amino acids, vitamins, and proteins, which will be discussed later.

The nonspecific nature of the antagonistic action of PAB is strikingly demonstrated by the following cases where the specificity of the relationship between substrates and their enzymes is well defined, and therefore is in no way related to the metabolic function claimed for PAB. In the following, PAB functions either as an inhibitor or as a nonspecific antagonist to the inhibitors: (a) the inhibition by PAB (as antioxidant) of the coupled oxidation of butter yellow (54); (b) the inhibition by PAB of bacterial luminescence (74a, 74c), carboxylase (157), and formic dehydrogenase (157) in a manner similar to that exercised by a sulfonamide; (c) the inhibition by PAB of the oxidative destruction of adrenaline in a tyrosinase-adrenaline system (116); (d) counteraction by PAB of the inhibitions of cholinesterase by sulfanilamide (218); (e) counteraction by PAB of the inhibition of bacterial carboxylase by sulfonamides (157); and (f) counteraction by PAB of the inhibition by sulfanilamide of the hydrolysis of starch by diastase, and of the adsorption of methylene blue on charcoal (43).

There are also several isolated observations which emphasize the nonspecific nature of the antagonism exercised by PAB. These are: (a) the counteraction by PAB of the protection exercised *in vivo* by sulfonamides against the toxicity of *Shigella* exotoxin and *Salmonella* endotoxin (72, 217); (b) neutralization by PAB of the toxicity, but not the trypanocidal activity, of arsenicals (145); and (c) shielding by PAB of bacteria against germanin and arsenicals in growth experiments (123).

In agreement with previous observations, Harris (59a) has reported that the most conspicuous and consistently present renal lesion caused by "tryparsamide" is characterized by the necrosis of convoluted tubules. In most tubules, nuclei had completely disappeared, and the cytoplasm was hyaline, eosinophilic, and homogeneous. In contrast, the kidneys of rats treated with PAB and "stibosan" or "tryparsamide" were normal or showed little necroses, but many mitoses. Because the extent of regeneration was usually less among the PAB animals, a consequence of less severe and less extensive tubular injury, Harris considers it *improbable that PAB caused an acceleration of regeneration*

with complete restoration to a normal status. In view of these results, it would seem that PAB combines with the sensitive site and thereby blocks the toxic actions of arsenicals, without exercising a metabolic effect on the sensitive system.

**Inhibition of Growth of Bacteria, Molds, and Viruses.**—In line with the observation that the antagonistic and various other actions of PAB are related to the inhibitory action it exercises, are the inhibitions of growth of bacteria, molds and the *Rickettsia* virus. The following are a few of the bacteria inhibited by PAB: *Photobacterium phosphoreum* (74a, 74c), *Streptobacterium plantarum* (85), *E. coli* (59, 65, 67), *Clostridium acetobutylicum* (144), *Mycobacterium tuberculosis* (177), and *Bacterium tularense* (186).

Cavill and Vincent (19) reported that PAB and its *n*-butyl esters inhibited the growth of *Aspergillus*, *Penicillium*, and *Byssoschlamys* on Czapek-Dox agar medium at pH 7.4. At a 5.6 millimolar concentration of PAB, the growth of *Aspergillus* was inhibited 50%, and at 1.0 millimolar concentration of the butyl ester of PAB growth was inhibited 70%. As low a concentration of PAB and butyl ester as 0.18 and 0.14 millimolar, respectively, gave observable inhibition.

After referring to other reports regarding the inhibitory action of PAB, Cavill and Vincent suggested that compounds like sulfanilamide and PAB might profitably be regarded as members of the same broad biochemical group possessing a similar "pattern" of inhibitory properties, showing quantitative rather than qualitative differences in their behavior.

The following brief summary of a study by Sciarini and Nord (151) on the mechanism of the action of *p*-aminobenzoic acid on the mold enzyme system illustrates the inhibitory action of PAB. In agreement with the observations of Oxford (129) on *Pseudomonas mors-prunorum* and of Cavill and Vincent (19) on other molds, Sciarini and Nord reported that, in the presence of potassium nitrate,  $1.46 \times 10^{-3}$  M of PAB inhibited the growth of *Fusaria*, as well as the fermentation of glucose. The inhibition of xylose fermentation by the same concentration of PAB was less marked, and, after the third day of analysis, inhibition of xylose fermentation disappeared altogether.

Inhibition by PAB was accompanied by an increased accumulation of nitrite (derived during growth from the enzymic reduction of nitrate ions), which indicated that the reduction of nitrite to  $\text{H}_2\text{N}-\text{OH}$  by HCN-insensitive mold reductase and dehydrogenases was blocked. Evidence was produced to the effect that this blocking by PAB occurred without any apparent alteration of its structure. In the presence of PAB the amounts of pyruvate produced from the fermentations of glucose and xylose were larger than the quantities detectable under normal conditions.

This increased accumulation of pyruvate resulted from the inhibition of the reduction of  $\text{NO}_2$  to  $\text{H}_2\text{N}-\text{OH}$ . The former, thus accumulated, reacted with the  $\text{NH}_2$  group of carboxylase, causing its inactivation, which was the reason for the nonutilization of the intermediary pyruvate.

The accumulation of  $\text{NO}_2$  in the culture fluid was demonstrated by the formation of the diazonium compound (from added PAB) and its coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, yielding a highly colored dye. The inhibitory action of PAB on the carboxylase system, as described above, is the direct result of the inhibition of  $\text{NO}_2$  reductase of the mold, which is different from its direct inhibitory action on bacterial carboxylases studied by Sevag *et al.* (157). A comparison of these activities of PAB emphasizes the nonspecific nature of its inhibitory actions.

Similarly, PAB has been found to inhibit the multiplication of *Rickettsia* virus in yolk sacs (52a). PAB in single injections of 1:6000 to 1:20,000 concentrations exercised rickettsiostatic, as well as synergistic, activity in combination with penicillin. In comparison, benzoic or *o*- and *m*-amino-benzoic acids were found to be ineffective. Furthermore, in clinical experiments, it was previously shown that PAB exercised a definite beneficial effect on the course of louse-borne typhus infection (215). In all these instances, it can be seen that PAB acts as an inhibitor of enzymes; in this role, it is also capable of counteracting the action of drugs and other toxic agents.

**Positive Effects of Enzyme Inhibitors.**—The inhibition by PAB of various enzyme systems and of bacterial growth is often erroneously explained by assuming that certain growth factors may exercise inhibitory action. As will be discussed below, this dual role is, in general, more often a common property of enzyme inhibitors, such as cyanide, azide, zephiran, narcotics, etc., which have been observed to stimulate respiratory enzyme systems at lower concentrations. Kuhn *et al.* (85) reported that the growth of *Streptobacterium plantarum* is inhibited by PAB when its concentration is twice that of the growth-stimulating concentration. They found that sulfanilamide likewise inhibits growth at higher, and stimulates it at lower, concentrations. Sulfonamides have been observed to stimulate growth in numerous instances (64a). PAB stimulates the growth of *Clostridium acetobutylicum*, but it inhibits growth when the optimal concentration is increased by two- to tenfold. Kimmig (80) reported that 0.00146 to 0.00073 *M* PAB inhibited, and 0.000073 to 0.000021 *M* stimulated, the growth of gonococci.

Dixon (31) reported that the amount of glucose utilized by rabbit cortex increased by 250% in the presence of 0.002 *M* sodium cyanide, while respiration was diminished 50%



and aerobic lactic acid production was greatly stimulated. Stone (184) showed that the lactic acid content of brain increased during cyanide convulsions and suggested that convulsions may be associated with increased tissue activity.

Lardy and Phillips (91) reported that maleate, hydroquinone, and potassium cyanide inhibited the respiration but stimulated the glycolysis by bovine spermatozoa. In a study on the effect of enzyme inhibitors and activators on the multiplication of typhus *Rickettsiae*, Greiff and Pinkerton (52) found that, in all experiments in which only minimal infection developed in the controls, a marked enhancement of rickettsial growth occurred as a result of the injection of potassium cyanide. They concluded that potassium cyanide lowers the metabolic rate of host cells by inhibiting cytochrome oxidase whereby the growth of *Rickettsiae* is stimulated. Similarly, rickettsiostatic action resulting from temperature elevation was neutralized by KCN. Apparently, the accelerated cytochrome oxidase activity, at elevated temperature of the host reticuloendothelial cells creates an unfavorable environment for rickettsial growth and is inhibited by potassium cyanide, thereby permitting multiplication of the parasite.

Sevag and Ross (159) found that zephiran in low concentrations stimulated, and in higher concentrations inhibited, the oxygen uptake of yeast cells. Sevag, Henry, and Richardson (unpublished data) found that 0.01 *M* sodium azide measurably inhibited, and 0.0001 *M* stimulated, the oxygen consumption of dysentery bacilli.

The principal point to be derived is that the stimulative effect of inhibitors is associated with an inhibitory effect on a simultaneously occurring enzyme system. As will be seen from the results of Sciarini and Nord, the reduction of  $\text{NO}_2$  to  $\text{H}_2\text{N}-\text{OH}$  is inhibited by PAB, resulting in the accumulation of pyruvate. In other cases, inhibitions are without similar simultaneous positive effects.

In contrast, in bacterial growth systems, the concentrations of vitamins may be increased 1000- to 20,000-fold without observing any inhibitory effect. In our experience increasing thiamin and nicotinamide concentrations 1000- to 10,000-fold failed to inhibit or reduce appreciably the growth of staphylococci in casein hydrolyzate. The results by Teply *et al.* (193) showed that a 20,000-fold increase in the concentration of nicotinic acid, even in the presence of 2  $\mu\text{g.}$  of sulfapyridine per ml., did not exercise any inhibitory effect on the growth of *Lactobacillus arabinosus*. Rantz's (137) studies with *Staphylococcus aureus* showed that maximal growth was obtained with 1  $\mu\text{g.}$  of nicotinic acid per ml. casein hydrolyzate, and 1000  $\mu\text{g.}$  per ml. failed to prevent maximal growth. The addition of  $1 \times 10^{-8}$   $\mu\text{g.}$  per ml. of thiamin chloride (or cocarboxylase) yielded optimal growth and a concentration of 100  $\mu\text{g.}$  per ml. (a 10-millionfold increase) failed to decrease below that of the optimal growth of *S. aureus* (or of *E. coli*). Only in the presence of 1000  $\mu\text{g.}$  per ml. (100-millionfold increase) of thiamin was the rate of growth decreased. Riboflavin, in concentrations up to 200  $\mu\text{g.}$  per ml. and pyridoxine, in concentrations up to 1000  $\mu\text{g.}$  per ml. medium, showed no effect on either organism. Pantothenic acid, in concentrations up to 200  $\mu\text{g.}$  per ml. medium containing excess thiamin and nicotinic acid, increased the rate of multiplication over that in its absence. In these experiments, parallel tests with PAB, in concentrations up to 100  $\mu\text{g.}$  per ml., failed to enhance the growth of either organism. In experiments with *Leuconostoc mesenteroides* p-60, a 1000-fold increase in the concentration of pantothenic acid had no inhibitory effect on growth (170).

In the majority of cases, it is not known precisely in what manner inhibitors of enzyme systems stimulate these or other enzyme systems. It may be that any inhibitor which is capable of undoing the work of other inhibitors is responsible for their stimulating effects, and may, in this manner, superficially appear to function similarly to growth factors, coenzymes, or specific substrates. Substances capable of counteracting the following inhibitions may represent this class: (a) the inhibition or inactivation of carboxylase by acetaldehyde produced from the decarboxylation of pyruvate (163); (b) oxidation of oxalacetic to malonic which strongly inhibits succinoxidase (199); (c) oxidation of amines by amine oxidases, producing aldehydes toxic to the enzyme systems which produced them (135); (d) inhibition of xanthine oxidase by the oxidation products of purines (154); (e) inhibition of  $\beta$ -hydroxybutyric acid oxidase by the reaction products of acetoacetic acid and structurally related compounds (154); (f) inhibition of proteolytic enzymes by the polypeptides, hydrolytic products of digested proteins (154); (g) inhibition of  $\beta$ -sugars resulting from the hydrolysis of soluble starch (154).

Neutralization, and, thereby, restoration of the normal functions of the inhibited enzyme system, is illustrated by the following examples: (a) the antagonism of N-allylnormorphine to morphine (195); (b) antagonism among amines in amine oxidase systems (135); (c) formation of cyanhydrins by the action of cyanide on acetaldehyde to prevent the inhibitory action of aldehyde on carboxylase, and of  $\alpha$ -keto acids on lactic dehydrogenase; (d) antagonism of malonic acid to the toxic action of quinoids or oxidized glutathione on succinoxidase (134); etc. In this connection, a suggestion by Mann and Quastel (114) may be of interest. They observed that the rapid inactivation of diphosphopyridine nucleotide by brain and liver tissue (perhaps due to the action of nucleotidase) was inhibited by nicotinamide, and they drew attention to the possibility that the therapeutic effects of nicotinamide may be due partially to its protective action on diphosphopyridine nucleotide breakdown.

Kensler *et al.* (78) found that addition of thiamin to alkaline-washed yeast carboxylase system increased the decarboxylation of pyruvic acid. This effect was most marked when low cocarboxylase concentrations were used. When high concentrations were used, thiamin had no stimulating effect on the reaction. In agreement with the explanation offered by Lipton and Elvehjem (101), Kensler and co-workers stated that both thiamin and cocarboxylase combine with catalytically inactive proteins and that the combination of these proteins with cocarboxylase deprives the alkaline-washed yeast carboxylase system of its coenzyme. On the other hand, the preliminary saturation of catalytically inactive proteins with thiamin leaves all added cocarboxylase to combine with the specific protein of the carboxylase system. In the presence of a large excess of

cocarboxylase, all the inactive proteins are saturated, leaving enough free coenzyme to conjugate with the catalytically active specific protein. In this case, the addition of thiamin would fail to bring about a stimulation.

Thus, catalytically inactive proteins function as inhibitors of enzyme systems, and the stimulating effect of thiamin under these conditions involves, not a direct action on the specific carboxylase system, but the neutralization of the inhibitor proteins. It is to be noted that when Cajori (16) used a purified carboxylase (apparently free from inhibitor protein), thiamin or cocarboxylase did not show any stimulating effect. That the foreign proteins, such as isolated serum albumin and globulins, combine with bacterial carboxylase systems, causing their inhibition, and that neopeptone is capable of antagonizing these inhibitions, was shown by Sevag *et al.* (158).

Sevag, Shelburne, and Ibsen (161) observed that inhibition of crystalline liver catalase by hydroxylamine and *p*-hydroxylaminobenzene sulfonamide is reversed by crystalline serum albumin. Apparently an albumin-inhibitor complex is formed, removing the inhibitor from catalase protein. These inhibitions are likewise reversed by hemin. In this reversal, the antagonist, hemin, seems to displace the inhibitor from the enzyme without exercising an inhibitory or accelerative effect on it. In a study of the role of various porphyrins in relation to the hematin requirement of *Hemophilus influenzae*, Granick and Gilder (49a) observed that iron-free porphyrins lacking vinyl groups and iron porphyrins compete for the porphyrin proteins which are the catalysts which make oxygen available to the organism. A porphyrin of ubiquitous distribution that might compete for the catalyst proteins and thus act as inhibitor is coproporphyrin. Granick and Gilder suggest that such a porphyrin would thus be a natural inhibitor, and, in a sense, a regulator governing the degree of anaerobic *vs.* aerobic metabolism.

It is possible that the growth-stimulating effects of PAB or similar substances, which, as stated above, do not possess catalytically reactive groups such as certain coenzyme groups, may be due to a mechanism involving the neutralization of inhibitory metabolic products.

In the absence of precise experimental observations, it cannot at present be said to what extent the above observations are capable of explaining the occurrence or abolition of lag periods during bacterial growth, or to what extent they are related to the stimulation of growth by preformed substances whose chemical natures are not suggestive of the ability to undergo reversible chemical changes in a manner comparable to those experienced by amino acids, carbohydrate metabolic products, and known coenzyme or growth factors. However, one must not lose sight of the importance of

such reactions in finding answers to the questions under investigation. A suggestion by Lodge and Hinshelwood (103) may be of interest. They believe that the stationary phases in bacterial growth are caused either by the exhaustion of food or by the accumulation within the cell of substances inhibitory to growth. According to them, after the initial utilization of foodstuffs, inhibitors are produced which prevent their further utilization, but not the formation of useful intermediates. As soon as the inhibitor-removing reaction comes into play, the intermediates are utilized again and give rise to an abnormally high growth rate until the normal rate is restored. These inhibitors were removed by oxidative aeration. See Part IV for discussion of the factors controlling induction periods.

### 5. Comments and Conclusions

On the basis of the results and observations discussed thus far, the following conclusions can be drawn. Competitive reactions between various inhibitors and antagonists show that, in many instances, the antagonists are structurally related to the inhibitors. However, in many cases, they are not. Except in a few instances, PAB exercises an antagonistic action to sulfonamides and other inhibitors merely as an inhibitor and not as a growth promoter. This is borne out by numerous observations showing that PAB inhibits the multiplication of certain *Rickettsiae*, bacteria, and molds, and various enzyme systems which bear no specific relationship to PAB as a metabolite.

Analysis of numerous inhibitor/antagonist molar ratios has revealed that high ratios are indicative of the action of antagonists, not as growth factors, but as competitive inhibitors. In contrast, low molar ratios, often less than unity, are indicative of growth-promoting effects by the antagonists. High ratios indicate an interference by the inhibitor with synthesizing processes, and lower ratios, an interference with the utilization of the preformed growth factors.

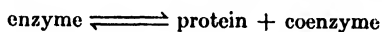
The studies on competitive reactions simply show that both inhibitors and antagonists compete for the same site in the catalytic system. These studies do not, however, reveal the nature of the specific catalytic systems involved. The exact mechanism, therefore, of the action of drugs remain undetermined. An understanding of the precise mechanism of drug action requires more than a study of competitive reactions. In the discussion which follows, the results of studies which offer answers to specific questions will be presented.

### III. Inhibition of Bacterial Respiratory Enzymes by Sulfonamides

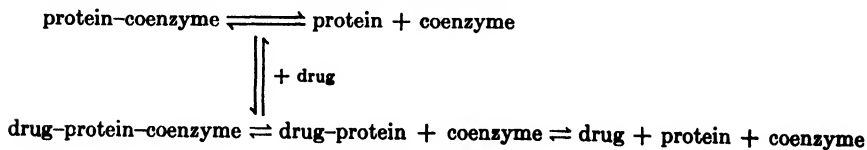
A bacterial cell must metabolize foodstuffs to derive energy and materials for the synthesis of essential cell components, such as vitamins, purines, pyrimidines, nucleic acids, carbohydrates, lipides, amino acids, proteins, enzymes, etc. Since all cellular functions need energy, oxidative reactions, mediated by respiratory enzymes, are involved in all phases of biology. The respiratory enzymes therefore occupy a central position in life. Sulfonamides could be effective by inhibiting bacterial respiratory enzymes and, thereby, the synthesis of essential cell components. The synthesis or the utilization of *p*-aminobenzoic acid by pathogenic bacteria has not as yet been critically demonstrated. However, assuming that it is a physiological factor, its relation to the basic processes of bacterial cells should not represent an exceptional situation. As with other cell components, its synthesis would depend on the uninterrupted functioning of the oxidative-reductive enzymes. The synthesis, therefore, of PAB would not differ essentially from that of, say, tryptophan, phenylalanine, tyrosine, etc.

The assumed functions of PAB and its antisulfonamide action fall, therefore, within the sphere of the activities of oxidative-reductive enzymes. The presence or absence of the inhibition of these enzymes by drugs would constitute the primary basis upon which the concept of the mechanism of the action of sulfonamides could be formulated.

The respiratory enzymes form dissociable complexes:



The coenzyme groups are related to thiamin, riboflavin, nicotinamide, and adenylic acid, the latter is the coenzyme of transphosphorylase, phosphorylase, as well as an essential component, respectively, of cozymases I and II, and alloxazine dinucleotide. Neither the coenzyme component nor the specific protein of the enzyme complex *per se* is considered capable of functioning alone. Combined with its specific protein, a coenzyme interacts with the activated hydrogen atoms of the foodstuff molecules and undergoes reversible oxidation-reduction. Sulfonamides or similar antibacterial agents, to be effective, must block one or several enzyme links in the chain of oxidative reactions. The possible ways by which antibacterial agents can exercise these effects may be presented in the following scheme:



With the above relationships before us, reference can now be made to results observed by various investigators, showing that sulfonamides interfere with:

- (a) utilization of glucose (22, 32a, 33, 48, 157, 160);
- (b) dismutation of pyruvate (157, 162, 163);
- (c) oxidation of glycerol, lactate, and pyruvate (111, 112);
- (d) oxygen uptake in the presence of succinic acid (48);
- (e) oxidation of glucose by *E. coli* to formic acid (but not the decomposition of the latter) (157);
- (f) oxygen uptake in the presence of glutamic acid (48);
- (g) synthesis and utilization of essential amino acids (64a) and tryptophan (155);
- (h) indophenol oxidase (13); and
- (i) inhibition of that fraction of cellular respiration (sea urchin egg) specifically concerned with providing energy for cell division (46, 64a).

The following observations may have some bearing on the relationship between the action of sulfonamides and antibiotics. In agreement with the findings of Sevag *et al.*, and in contradiction to those of Wyss *et al.* (214), Schuler (149) reported that sulfathiazole had no inhibitory action on the respiration of resting staphylococci. Neither did he observe any effect with highly purified penicillin. In contrast, the oxygen uptake and the evolution of glucolytic carbon dioxide by these cells during growth were strongly inhibited. Chain and Duthie (20) likewise reported that, during the resting phase, even large concentrations of penicillin had no effect on the rate of oxygen uptake by staphylococci. During the early lag and logarithmic phases of multiplication, penicillin (0.04 to 0.1 unit per ml.) exerted a strong inhibitory effect on, and eventually completely stopped, the oxygen uptake of the bacterial suspension. Chain and Duthie concluded that penicillin appears to interfere with a metabolic function involved in the early stages of bacterial development. Comparing their results with those of Hirsch (67), they agree that penicillin has no effect on the respiration of resting bacteria, but that it does affect that of growing bacteria in a characteristic manner. They disagree, however, with Hirsch's contention that penicillin acts on bacteria only during the phase of active multiplication and that bacterial growth always continues at its normal rate for some time after the addition of penicillin.

Happold and Waters (57), using animal tissues, or washed suspensions of *Escherichia coli* or *Pseudomonas pyocyaneus*, studied, by means of the Thunberg and Bancroft apparatus, the effect of clavatin on respiratory enzyme systems. Complete inhibition of the (>95) glucose, malic, and  $\alpha$ -glycerophosphate dehydrogenases, and of succinoxidase and tryptophanase was observed. Partially inhibited were mannose and lactic acid de-

hydrogenases, and *d*-amino acid oxidase. Little effect was observed on uricase and cytochrome oxidase. No inhibition whatsoever was observed on trypsin, pepsin, pancreatic lipase, catalase, intestinal phosphatase, and vegetable tyrosinase. Hotchkiss (71a) reported that, though gramicidin does not interfere with the respiration of staphylococci in glucose, it blocks the removal of inorganic phosphate from the medium. The phosphate uptake does not refer to increase in phosphate esters, but increase in total phosphorus sedimentable with the cells (as determined by the difference exhibited by the supernatant liquid). On the other hand, according to Blinnikova (8a), tyrothricin inhibits not only enzymic dehydrogenation of glucose, but also that of glutamic acid, ethyl alcohol, lactic acid, fumaric acid, and acetic acid. Dehydrogenation, and the growth of a particular microbe, were inhibited by exactly the same concentration of tyrothricin.

The results of *in vitro* studies dealing with the mode of action of sulfonamides and other antibacterial agents should be comparable with those obtained *in vivo*. This necessitates consideration of the role of some of the components of the *in vivo* environment. Thus, serum proteins, metabolites, protein degradation products, vitamins, and amino acids condition the action of drugs on bacteria. Sevag and Shelburne have shown (160) that the inhibitory effects of sulfanilamide on aerobic and anaerobic respiration of *S. pyogenes* in the presence of blood serum were proportional to the inhibition of growth, an effect which can be related to the efficacy of this drug in clinical practice. The inhibition of respiration and growth of pneumococci *in vitro* in the absence, and the lack of inhibition in the presence, of serum proteins (158) may similarly be related to the relative ineffectiveness of sulfanilamide against this organism in clinical practice. Sulfanilamide does not inhibit the aerobic or anaerobic oxidation of glucose by staphylococci in the presence or absence of serum proteins, which again may account for the therapeutic inefficacy of this drug against this organism (158). In the absence of serum proteins, the growth of staphylococci in restricted synthetic medium may be inhibited, but these inhibitions are abolished in the presence of serum, vitamins, etc. This shows that, in well-controlled respiration experiments, inhibition results can bring out true sulfonamide activity, provided the above-mentioned and other factors are kept in the background. Wyss *et al.* (214) reported a study from which they concluded that studies on inhibition of respiration do not show true sulfanilamide activity. Since these investigators experimented in simple buffer media, their conclusion may, no doubt, be accurate as far as the conditions they used are concerned. Their results neither resemble those obtained by Sevag and Shelburne, nor do they serve to bring out true sul-

fonamide activity. As discussed above, the factors controlling sulfonamide activity must be taken into consideration to avoid inadequate conclusions.

In our experiments, designed to correlate the inhibition of gaseous exchange with the growth of bacteria in a given system, it was necessary to use as inoculum a large number of cells to produce a metabolic condition whereby the volume of oxygen consumed or carbon dioxide evolved could accurately be measured. Under these conditions, the amount of sulfonamides used per milliliter of medium was of necessity greater than the amount to be found in infected blood during sulfonamide treatment. This difference might, superficially, give occasion to presumptions (136a) that this high concentration of drug *in vitro* might inhibit bacterial sites different from those inhibited *in vivo* with smaller amounts of sulfonamide. Let us analyze the data to learn the actual facts.

In our studies with *Streptococcus pyogenes* (160), for example, the inoculum at 0 hours corresponded to  $118 \times 10^{-3}$  mg. of streptococcal nitrogen or  $2.44 \times 10^9$  cocci in a volume of 5.4 ml. containing 0.04 M sulfanilamide, or  $6.43 \times 10^7$  cocci per mg. drug per ml. medium. Under these conditions, a 64% inhibition of oxygen consumption caused practically complete inhibition of bacterial multiplication. The relationships in our subsequent studies do not deviate from this by a factor of more than about 3.

According to findings of various investigators (6c, 19a, 95a), in septicemia, the number of colonies of streptococci, pneumococci or staphylococci, etc. might range from 1 to 2000 per ml. blood. According to the hospital records of Dr. S. Brandt Rose of Philadelphia General Hospital, the numbers of colonies obtained in blood cultures are very much lower than 2000 per ml. However, let us take the highest figure of 2000 colonies per ml. blood. During sulfonamide treatment, the level of drug for sulfanilamide is from 5 to 6 mg. per 100 ml. blood (for sulfathiazole, from 8 to 10 mg. per 100 ml.), or 0.05 to 0.06 mg. of sulfanilamide per ml. blood. This gives a relationship of  $2 \times 10^4$  cocci per mg. sulfanilamide per ml. blood. When we contrast this value with the above  $6.43 \times 10^7$  cocci per mg. sulfanilamide per ml. metabolic system, we have a difference of nearly 3200-fold. In other words, 1 mg. sulfanilamide is capable of producing an antibacterial effect on a 3200-fold greater number of cocci than *in vivo*. This difference might be due in great part to the competition between bacterial and body proteins (and vitamins) for the drug. These considerations fail to support presumptions (136a) which might arise from an inadequate consideration of our experimental conditions.

**Limitations When Oxygen Alone Is Measured.**—It must also be kept in mind that, in experiments which attempt to demonstrate the relation of the inhibition of respiratory enzymes by sulfonamides of oxygen uptake to their activity on bacterial growth, measurement of oxygen uptake alone is not always adequate. After all, oxygen is one of numerous hydrogen acceptors participating in the respiratory metabolism of living and growing cells. If the organism can utilize amino acids or other intermediary metabolites without oxygen as hydrogen acceptor, inhibition cannot be detected by measuring the oxygen uptake and must be determined by other methods (158, 159). Perhaps this is the reason that various workers fail to observe a relationship between the inhibition by sulfonamides and similar agents of respiratory enzymes and the growth of bacteria (35c).



In this connection an observation with sea urchin egg is of interest (46). Sulfonamides inhibit, not the over-all, total respiration of the cell, but only that fraction which is specifically concerned with providing energy for cell division. Sulfonamides and narcotics inhibit this fraction more or less specifically, thus accounting for complete inhibition of division by a relatively small inhibition of respiration. Henry and Henry (64b) found that penicillin (250 to 2650 units per ml.) inhibited the rate of division of the fertilized sea urchin egg from 0 to 100% without any effect on the oxygen consumption of the fertilized or unfertilized egg.

There is, of course, no information available from measurements of oxygen consumption regarding the inhibition of respiratory mechanisms in which oxygen does not participate but which, nevertheless, may play a significant role in cell division. This may be one of the reasons that, in studies with penicillin, the inhibition of oxygen uptake is variously contested. It is possible that during the lag or logarithmic phase, of bacterial growth, or the periods preceding them, anaerobic oxidation-reduction reactions are inhibited by penicillin, but that adequate methods are not employed to determine these effects.

### 1. *Role of Respiratory Enzymes in Synthesis of Essential Metabolites*

On the basis of the results of the above discussed study by Wyss *et al.* (214), van Niel (198) declines the *inhibition of the respiratory enzyme theory* advanced by us (158, 160, 162). Dubos (35c) considers this discrepancy and indecisively poses the question of

"... whether the primary effect of the sulfonamides is to block the respiratory processes of susceptible organisms and thus cause inhibition of growth (Sevag and Shelburne, 1942), or whether the drugs prevent the synthesis of some essential metabolite or compete with it for a given enzymic system and affect respiration only indirectly (Van Niel, 1943b; Wyss, Strandkov, and Schmelkes, 1942). In any event, it is clear that, unlike the detergents, the phenols and many other types of general protoplasmic poisons, the sulfonamides do not cause a complete inhibition of oxygen uptake and acid production."

We have already pointed out above that oxygen is only one of the numerous hydrogen acceptors which take part in oxidative reactions. Failure to observe a complete inhibition of oxygen uptake does not indicate a failure of inhibition of respiratory processes. Failure to comprehend these relationships would lead to erroneous conclusions.

This quotation from Dubos (35c) also gives the impression that "... the synthesis of some essential metabolite ..." is a process distinct from the oxidative-reductive reaction, or is not dependent on the chemical reactions mediated by the respiratory processes. Such an impression would be mis-

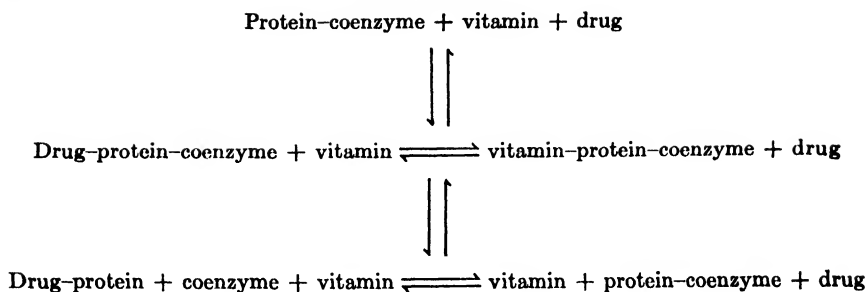
leading. As is well known, the essential metabolites, amino acids, vitamins, purines, pyrimidines, etc., some of which are utilized in the synthesis of coenzymes and prosthetic groups of conjugated proteins, are synthesized principally by oxidation-reduction reactions, or are mediated through the respiratory processes.

In a growth-supporting simple medium, made, for example, of an ammonium salt as source of nitrogen, glucose as source of carbon, salts, and buffer, bacteria must metabolize glucose to liberate the required energy as well as to yield  $\alpha$ -keto acids, aldehydes, fumaric and other acids which are essential to the synthesis of amino acids, etc. The liberation of these oxidative intermediary and end products requires the unobstructed functioning of the respiratory enzymes. The synthesis of amino acids, whether by (a) direct amination of an  $\alpha$ -keto acid, (b) by coupled oxidative-reductive reactions involving  $\alpha$ -keto acids and ammonia, or (c) by transamination involving the intermolecular oxidation-reduction of an  $\alpha$ -amino acid and  $\alpha$ -keto acid, involves a mechanism which is mediated by respiratory enzymes, probably flavoproteins. Following the synthesis of amino acids, or simultaneously with it, occurs the elaboration of vitamins, nucleic acids, lipides, etc. The structures of these compounds indicate the probable steps involved in their synthesis. Ring closures, involving dehydrogenation reactions and leading to the formation, for example, of pyridine, alloxazine, vitamins, coenzymes, etc., result principally from oxidation-reduction reactions.

Since the proteolytic enzymes which catalyze hydrolysis and synthesis of proteins are not inhibited by sulfonamides, and since respiratory processes, essential to the synthesis of higher complexes, are inhibited by sulfonamides, an experimental basis for the quotation from Dubos does not appear to exist. The reasonable conclusion seems to be that the inhibition of respiratory enzymes is the basis for the mechanism of the antibacterial action of sulfonamides, and, probably, of antibiotics.

## *2. Vitamins and Antibacterial Action of Sulfonamides*

The inhibitory action of sulfonamides on bacteria is the result of a reversible combination of the drug with the specific proteins of bacterial respiratory enzymes. Coenzymes, which are derivatives of certain vitamins, also form reversible combinations with these same enzyme proteins. There is thus antagonism between certain vitamins and sulfonamides. The result of this antagonism depends on the concentration and the relative degree of affinity of the competing substances for the enzyme protein.



It is known that most pathogenic (or parasitic) bacteria are incapable of synthesizing vitamins; they are dependent upon the vitamins of the host for the synthesis of their respiratory enzymes and thereby for their multiplication. Sulfonamides must necessarily prevent the utilization of these vitamins and, thereby, the multiplication of the organism. However, as indicated in the diagram, vitamins may win out over sulfonamides and thus render them ineffective against the bacteria. Experiments have shown that nicotinamide (32a, 33), thiamin (41), cocarboxylase (163), riboflavin (155), and pantothenic acid (155, 204, 205) completely counteract sulfonamides. Certain clinical observations appear to support these results. Perhaps, then, it might be beneficial to keep the vitamin intake of the patient at a minimum during sulfonamide treatment.

### 3. Relation of Species and Enzymic Specificities to Action of Sulfonamides and Other Drugs

In an analysis of the specific action of drugs, it is necessary to consider the species specificities of various microorganisms as well as the specificities of their proteins. Sulfonamides exercise selective action on different species of bacteria and certain viruses by virtue of their selective affinity for specific enzyme proteins of different species. The action of sulfanilamide against *Streptococcus pyogenes* and the relative inaction against *Pneumococcus* and *Staphylococcus* may thus be explained. Furthermore, the various respiratory enzymes of a given strain exhibit selective affinities for sulfanilamide and *vice versa*. For example, sulfanilamide was found by us to inhibit the oxidation of glucose to formic acid by *Escherichia coli*, but it failed to inhibit the formic hydrogenlyase involved in the decomposition of formic acid to hydrogen and carbon dioxide (157). That is, sulfanilamide differentiated between two enzymes within a given cell. With *Pneumococcus* type I, it has been observed that sulfapyridine failed to inhibit the oxidation (Thunberg methylene blue technique) of glucose (111, 112). On the

other hand, the drug inhibited the oxidation of glycerol, lactate, and pyruvate, showing a selective affinity for the protein components of the particular enzymes involved in the oxidation of these substrates. Similarly, it has been reported that sulfanilamide inhibited the cytochrome-cytochrome oxidase system of *Gonococcus* but had no action on its catalase and peroxidase (13). These three enzymes possess identical heme groups, but differ in their protein components, which may explain the selective action of sulfanilamide on cytochrome-cytochrome oxidase.

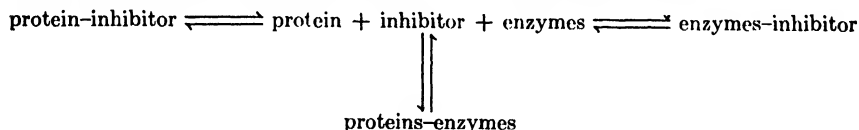
In a study on isolated enzyme systems from purified yeast and from tissue, Haas (55) found that atabrine inhibited cytochrome oxidase three times as effectively as did quinine. However, the inhibition was considered too small to account for the effect of atabrine on the respiration of malaria parasites. Atabrine failed to inhibit the activity of cytochrome c. In contrast, atabrine interfered strongly with cytochrome reductase (a mononucleotide flavoprotein). This inhibition was comparable in degree to that exercised on malaria parasites. Atabrine also interfered strongly with a crude preparation of tissue flavoprotein capable of oxidizing *d*-amino acid (211). These results show that atabrine, which is structurally unrelated to the prosthetic groups (heme) of cytochrome c and cytochrome oxidase, failed to markedly inhibit the specific proteins of these enzymes. On the other hand, the drug, being structurally related to the coenzyme groups (alloxazine mono- and dinucleotides) of flavoproteins, strongly inhibited these enzymes. It has also been observed that trypaflavin (acriflavine) interferes, not with the cyanide-sensitive, but with the cyanide-insensitive, hydrogen transport components of the respiratory system of trypanosomes (147). Studying the specific effect of narcotics, Quastel (135) reported that they interfered with the flavoprotein link of tissue respiratory enzyme system. That is, they had no effect on the cozymase-dehydrogenase or cytochrome systems.

#### 4. *Competition between Bacterial Enzymes and Nonbacterial Proteins for Inhibitors*

The role of host proteins in regulating the efficacy of drugs against infection must also be considered. Sulfonamides act on bacteria by combining with their respiratory enzyme proteins, but they are also capable of preferentially combining with nonbacterial proteins. The competitive relationships already discussed may serve as a basis for explaining the efficacy, for example, of sulfanilamide in clinical practice and *in vitro* experiments in inhibiting the multiplication of *Streptococcus pyogenes* (160) in an environment containing blood components. Sulfanilamide exercises a greater affini-

ity for the bacterial enzyme proteins than for tissue and blood proteins. In contrast, the relative inefficacy of sulfanilamide against *Pneumococcus* and *Staphylococcus* (158) indicates a lesser affinity of the drug for the enzyme proteins of these bacteria and greater affinity for the body proteins. For it has been shown that sulfanilamide is capable of inhibiting the growth of the latter organisms in a medium devoid of blood proteins. In their presence, however, the organisms grow.

In this connection it may be noted that the *o*-, *m*-, and *p*-isomers of aminobenzenesulfonamide similarly inhibit bacterial enzymes in the absence of body proteins. In contrast, serum albumin (156, 158), abolishes inhibition by the *m*- and *o*-isomers but not inhibition by the *p*-isomer.\* Similar results have been obtained by Davis (29) with *E. coli*. It has also been shown that blood serum completely counteracts the inhibition by germanin (3) of an isolated carboxylase system. Blood serum completely counteracts the inhibition by zephiran of bacterial growth and of the cytochrome oxidase system (159). Dittmer and du Vigneaud (30) reported that the addition of imidazolidone caproic acid or imidazolidone valeric acid to yeast culture in the presence of avidin-bound biotin displaces some of the biotin which then becomes available for the growth of the organism. These relationships can be presented in the following manner:



That body proteins combine with bacterial enzymes has been shown in respiration experiments. The components of blood globulin, albumin, etc. combine with bacterial enzymes and thereby counteract the action of the inhibitors. Protein-enzyme combinations have been shown to be inhibitory (158), though such inhibition is not a necessary condition for the antagonism exercised toward the drugs.

\* From the standpoint of specific affinity between the host proteins and a given drug the following observations are of interest. Using a chemical method of analysis, Jendrassik (73a) reported the absence of affinity between the sera of cat, horse, or human for pilocarpine, and the presence of a strong affinity between rabbit serum and this alkaloid. In the presence of any of the above three sera, pilocarpine formed a thick brown mass with an alkaloid reagent (2.5% iodine in 5% potassium iodide). In contrast, in the presence of rabbit serum, this reaction did not take place. However, using a diffusion method of determining the affinity of pilocarpine for various sera, R. Beutner, working in Storm van Leeuwen's laboratory (184a), demonstrated a nondialyzable combination between 2 mg. pilocarpine and 5 ml. rabbit, beef, sheep, or human sera. In contrast, pilocarpine readily dialyzed out when it was in contact with a solution of egg white. Under these conditions, a specific affinity between the four sera and pilocarpine, but none at all between the alkaloid and egg white, was demonstrable.

That the affinities of host proteins for drugs play a determinant role in regulating the efficacy of drugs and disinfectants has been known for many decades. Ehrlich (37) refers to the surprise that Koch experienced when he found that while a small amount of mercuric chloride killed anthrax bacilli *in vitro*, a manifold amount of it had no effect on the bacilli *in vivo*. The animal, dead of mercury poisoning, contained surviving bacilli. Busk (15), experimenting with paramecia, found that sensitizing toxic effects of various substances were counteracted by blood serum. In contrast, glue, gum arabic, starch, and amphoteric substances were unable to neutralize these toxic effects. Ehrlich found that tetrabromocresol exercised, *in vitro*, an extraordinary killing effect on dysentery bacilli. But the injection of a 100-fold amount of this agent into an infected animal did not produce disinfection; it was likewise nontoxic to the host. On subsequent *in vitro* experimentation, he found that blood serum neutralized this substance. On the basis of these and similar observations, he concluded that the relative affinities of the host and bacterial proteins for disinfectants determine their antibacterial action.

#### 5. Comments

The mechanism discussed of the antibacterial action of sulfonamides poses the question of the nature of the ineffectiveness of these drugs against nonsusceptible organisms. In the case of *Staphylococcus aureus*, it appears fairly certain that the affinity of the drugs for these cocci is readily nullified by serum proteins. In other cases, however, we are, perhaps, dealing with problems of a different nature. Whether the enzyme proteins of nonsusceptible bacteria are accessible to the drug, but are devoid of any affinity for it; or whether the drug is unable to get at them for structural or mechanical reasons remains to be investigated by means of special techniques which hitherto have not been used.

### IV. Critique on "Adaptive" Enzymes

From time to time certain experimental results have been presented using the terms "formation," "production," or "synthesis" of "adaptive" enzymes. According to these reports, certain substrates stimulate the production or synthesis of enzymes in cells which previously lacked them completely, or were very markedly weak in that respect. This subject has been discussed before (35a-35c, 75) and will not be reviewed here. The principal part of these studies were carried out without eliminating individual organisms of different qualitative and quantitative chemical or enzyme activities. Using the single-cell isolation technique, and also testing

numerous colonies from ordinary plain agar plates, Lewis (95) carefully inquired into the nature of the lactose-fermenting ability of *E. coli*. He found that lactose-fermenting variants occur without regard to environmental influences, or rather in the absence of lactose in growth media. Under growth conditions, a selection of strains occurred rather than "adaptive" enzymes.

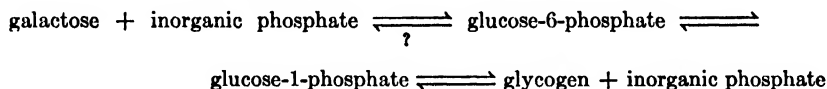
To meet these and similar objections to the idea of adaptive enzymes being formed by bacteria, it has lately been reported that adaptive enzymes are produced under conditions which do not permit multiplication, and even simply in salt-buffer suspensions containing the specific substrate. How synthesis of an enzyme of complex structure can occur in the absence of catabolic and anabolic processes is a puzzling question. Unfortunately, no critical chemical investigation has as yet been carried out on the nature and presence of essential factors in one or the other metabolic environment. The presence of such factors might account for these observations.

The concept of the adaptive nature of certain enzymes raises the substrate in question to the position of an unusual catalyst, though it is not used in catalytic amounts but usually in amounts greater than the substrate concentrations required for enzyme reactions. In other words, the substrate performs functions greater in scope than ordinary biocatalysts; for they catalyze the synthesis of adaptive enzymes which then catalyze the substrate. In this connection, it must be noted that the adaptive enzymes are "produced" only when the stimulating substrates are structurally related to substrates which the cells have the inherent ability to metabolize. Also, it must be noted that, in most cases, the cells, at one time or other, have exercised "adaptive" enzyme activities without having been adapted; or that the strains undergoing adaptation are species specifically related to those strains which readily metabolize these substrates. Cells falling outside of these requirements have not been shown to synthesize any adaptive enzymes, nor have the substrates been shown to stimulate their synthesis in these cells.

Besides the above considerations, there are other reasons for a critical analysis of this problem. One of these is the indiscriminate use of the term "adaptation" for "drug resistance." These two terms stand for two different or opposing processes. The former has thus far been associated with as yet experimentally unproved processes of building-up, with an increase or expansion of the biochemical activities of cells. In contrast, the latter term stands for a material loss, or loss of an activity under the impact of toxic agents. It would seem that a reorientation in our reasoning and application of these terms might eliminate confusion and contradiction.

# 1. *Is Galactozymase an Adaptive Enzyme?*

**Formation of Glycogen and Polysaccharides from Galactose and Glucose.**—The adaptive enzyme most widely studied is galactozymase. It may therefore be expedient to begin our inquiry with the fermentation of galactose. Galactose-1-phosphate has been reported to be present in the liver of rabbits and rats during the assimilation of galactose (82), and glucose-1-phosphate has been isolated from mammalian tissue (27). Glycogen has been synthesized reversibly by purified yeast phosphorylase (79), and by crystalline mammalian tissue phosphorylase (27) from glucose-1-phosphate. In animals, galactose (like glucose or fructose and mannose) is converted into glycogen (24), which on hydrolysis yields glucose but not galactose (58). Apparently, the formation of glycogen from galactose proceeds in the following manner:



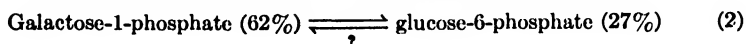
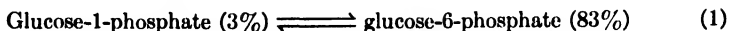
The conversion of galactose into galactose-1-phosphate would require a hexokinase or heterophosphatase. Since no enzyme is known which is capable of converting the other hexose-1-phosphates into glucose-1-phosphate, the above equilibrium reactions are a plausible explanation of the observed facts. This is corroborated by the following: The polysaccharides synthesized from galactose by yeast cells yield glucose upon acid hydrolysis (also fructose and mannose) but not galactose (50). Trehalose monophosphate, synthesized during the fermentation of galactose by yeast, upon hydrolysis by bone phosphatase yields *d*-glucose and *d*-glucose monophosphate, but not galactose. The same products are obtained during the fermentation of glucose, fructose, and mannose. These facts indicate a common path from various hexoses to the glucose structure during fermentation.

**Products of Galactose Fermentation.**—Glucose-6-phosphate (128a), fructose-1,6-diphosphate (50, 128a), trehalose monophosphate (50), and phosphoglyceric acid (18) have been isolated from the fermentation mixtures of galactose; these products have also been isolated from the fermentation of glucose, mannose, and fructose.

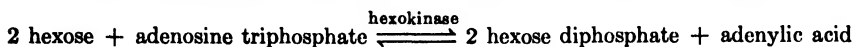
Kosterlitz (82) found synthetic galactose-1-phosphate to be as active as glucose-1-phosphate in yeast fermentation though no glucose-1-phosphate was found in the reaction mixture of synthetic galactose-1-phosphate; and there was no evidence of the occurrence of a galactose phosphate when free



galactose was fermented (50). The findings of Kosterlitz indicate the following states of equilibria:



In connection with the above results, it may be of interest to consider the role of the hexose phosphorylating enzyme. Meyerhof (119) isolated an enzyme from muscle which he called *hexokinase*, and which had the ability to phosphorylate hexose:



Using a yeast preparation, Euler and Adler (42) obtained an enzyme, called *heterophosphatase*, which transformed fructose into glucose-6-phosphate.\* Heterophosphatase is identical with hexokinase. Colowick and Kalckar (23) isolated this enzyme from yeast, in purified form, and found that it catalyzed the phosphorylation of glucose or fructose to the same glucose-6-phosphate. Magnesium ion was used in all these reactions as an activator. Hexokinase thus effects the transposition of labile phosphate from the coenzyme adenosine triphosphate to these hexoses, yielding a single end product. Colowick and Kalckar (23) found that the transformation of glucose-1-phosphate is not the primary reaction. The enzyme responsible for the reaction  $\text{glucose-1-phosphate} \rightleftharpoons \text{glucose-6-phosphate}$  was not present in the hexokinase.

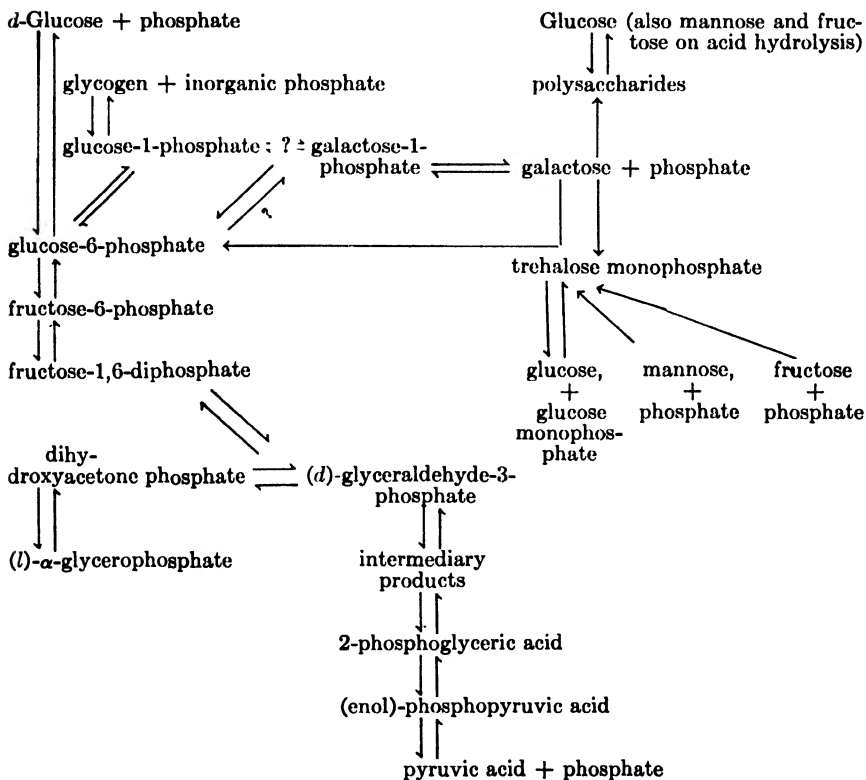
In the fermentation of galactose, the immediate link between the non-phosphorylated galactose and the intermediary reaction products appears to be glucose-6-phosphate. Neither glucose-1-phosphate nor phosphorylated galactose has been found in the reaction mixture. Thus the link from galactose to glucose-6-phosphate is probably a direct one, especially since in the fermentation of synthetic galactose-1-phosphate, about 62% has been found to be unaltered, and synthetic galactose-6-phosphate is non-fermentable (50, 82, 93).

The above considerations focus attention on yeast hexokinase as the single means of revealing the adaptive nature of galactozymase if any. For once galactose is transformed into glucose-6-phosphate the remaining course of the fermentation is common to all hexoses. In what manner does hexokinase bring about the intraconversion of the hexose molecule? A study by Robison (141) showed that the fermentation of mannose, glucose, and fructose by yeast juice yielded the same fructose-1,6-diphosphate. Galactose is also converted to this substance, as shown above, as well as to

\* This reaction takes place then even when an oxidation-reduction reaction does not occur in the system. However, according to Colowick and Price (23a), during the phosphorylation of glucose by muscle extracts to glucose-6-phosphate, or of fructose-6-phosphate to fructose-1,6-diphosphate in the presence of adenine triphosphate, both dihydrocozymase and guanine are essential coenzymes. In other words, the first step in the fermentation process involves oxidation-reduction reactions.

its precursor. The four hexoses at some stage in their phosphorylation pass through a form which is common to all. Robison presents a scheme of hypothetical structural modifications assumed to occur during the esterification of the hexose molecule. It may be that yeast contains a single hexokinase which phosphorylates the different hexoses nonspecifically at various carbons. Intramolecular rearrangement yields glucose-6-phosphate which appears to be the most desirable form of hexose for yeast fermentation.

A scheme of fermentation (Scheme I) has been prepared from facts previously known, as well as from the above experimental results.



SCHEME I

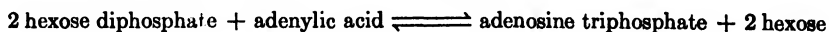
**Induction Periods of Fermentation.**—That the fermentation of galactose (and very frequently of glucose and other hexoses) is preceded by a long (sometimes indefinite) period of induction has been observed by vari-

ous investigators. Induction periods occur in fermentations by both "adapted" and "nonadapted" yeast cells. These induction periods have been ascribed to the time required for the synthesis of galactozymase. It is interesting that induction periods are abolished by treating the yeast with oxygen, or by adding hexose diphosphate to the fermenting system. An analysis of the function of these factors is of significance.

**Abolition of Induction Periods by Hexose Diphosphate.**—Levene and Raymond (93) reported that certain phosphorylated sugars abolish the induction period in the fermentation of galactose by zymine. Nonphosphorylated sugars show no such activity. The zymine used was prepared from an adapted bottom yeast, and exhibited an induction period of 90 minutes. By adding phosphorylated esters of glucose at a catalytic concentration of 0.005 mole per liter, the induction period was entirely abolished (hexose diphosphate) or reduced to 45 minutes (synthetic glucose-6-phosphate, Robison ester). Galactose-6-phosphate, on the other hand, acting as an inhibitor, increased the induction period from 90 to 110 minutes, and was nonfermentable. Kosterlitz (82) observed in all experiments, whether with glucose or galactose or their esters, an induction period and a slow fermentation (period of hexose diphosphate fermentation). He found that catalytic amounts of acetaldehyde and hexose diphosphate (Harden-Young ester) almost abolished the induction period in the fermentation of free galactose and glucose by a preparation of yeast (grown in 3% galactose), with simultaneous increase in the maximal rates of carbon dioxide production. Acetaldehyde scarcely influenced the fermentation of either the glucose-1- or galactose-1-phosphate esters, indicating that the abolition of the induction period was not due to a reaction between acetaldehyde and hexose monophosphates.

Hexose diphosphate, or some other phosphoric ester, is the critical substance in the elimination of the induction periods of yeast fermentation.

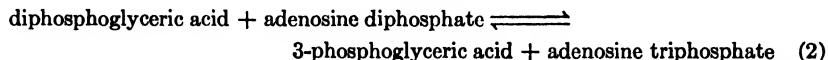
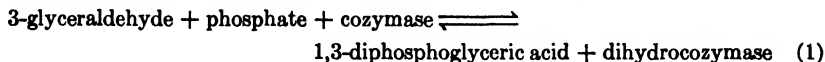
Hexose diphosphate functions as a catalyst in the sense that it donates phosphate to free hexose. This transposition of phosphate requires the functioning of heterophosphatase or hexokinase, of which the coenzyme group is adenylic acid (or adenosine triphosphate for cell-free hexokinase). Studies by various investigators (42, 104, 106, 121) have resulted in the following conclusions: (a) phosphorylation of hexose mediated by yeast hexokinase is independent of oxidation-reduction reactions; (b) phosphorylation of hexose involving oxidation-reduction reactions involves cozymase dehydrogenase. Either of these, alone or in combination, can eliminate the induction period of fermentation. Euler and Adler stated that in cell-free fermentation systems, added adenosine triphosphate eliminates the induction period. However, they found that hexose diphosphate was superior to the former in this respect:



Adenosine triphosphate formed in this manner transfers its labile phosphate to a hexose molecule.

As will be seen from the scheme of fermentation reactions, the oxidation-reduction reactions which may directly be responsible for the phosphorylation of hexoses do not begin until fructose-1,6-diphosphate splits reversibly into triose phosphate. From the

standpoint of the elimination of induction periods of fermentation, the following transphosphorylations concomitant with hydrogen transfer may be of interest (125, 200):



Why do yeast and yeast preparations require outside factors to initiate the fermentation of hexoses, even when the cells are claimed to have been adapted to galactose? The answer lies in the observation that the cells undergo autolysis, or sustain injuries or losses of enzymes during growth. According to Lutwak-Mann and Mann (106), in yeast juice and dry yeast, the amount of adenosine triphosphate and adenylic acid is very small. In the presence of a very small amount of adenosine triphosphate, and with the addition of hexose diphosphate, the fermentation starts very promptly.

According to Meyerhof (120), there are two successive phases of alcoholic fermentation by yeast extracts. First there is a rapid phosphate phase (when hexose diphosphate is present); then a second, slower phase during which either inorganic phosphate or free sugar is exhausted, depending on which was present in excess at the start. During this period, the fermentation rate is controlled by the fermentation of hexose diphosphate. In the absence of a phosphate acceptor, adenosine triphosphate can be split only by means of adenylypyrophosphatases, which are the most sensitive and the least extractable of all enzymes and which do not distinguish between adenosine di- and triphosphates.\* These two distinct phases of alcoholic fermentation in cell-free systems result from a destruction of the sensitive and structurally bound adenylypyrophosphatase of the living yeast by the drying and extracting procedure.

The induction period, or the inability of intact yeast cells to ferment sugar, may be due to a deficiency or destruction of any or all of the following: adenylic acid or adenosine triphosphate, adenylypyrophosphatase, and hexose diphosphate. In the case of glucose, fermentation during growth

\* In a study on the metabolism of spermatozoa, Mann (115) reported that when spermatozoa were incubated anaerobically without glucose, their content of adenosine triphosphate decreased rapidly, while in the presence of glucose it could be preserved much longer. This behavior was considered in agreement with the observation that when spermatozoa were stored anaerobically, motility was maintained more satisfactorily in the presence than in the absence of a glycolyzable substrate. The metabolism of glucose in the sperm is reported to be initiated by the shift of labile phosphate groups from adenosine triphosphate to glucose. The enzyme involved in this reaction was considered resembling the hexokinase of yeast. These conditions were said to prevail in aerobically glycolyzing spermatozoa as well.

In a carefully controlled study in connection with anaerobic glycolysis in brain, Utter, Reiner and Wood (197) show clearly the necessity of hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide, and magnesium chloride in order to obtain full activity with brain homogenate. These factors are subject to immediate attack after removal of the tissue from its natural environment, and especially after disruption of the cell. They point out that the previously reported alterations in anaerobic glycolysis during poliomyelitis, as measured with low activity preparations without control with respect to the above critical factors, are of doubtful significance in so far as the changes may be related to any specific phase of metabolism.

may completely exhaust some or all of these essential factors. A yeast obtained from such an environment would be incapable of fermenting galactose or would show markedly reduced activity, or a long induction period. This may be the reason that a yeast which has been adapted to galactozymase may be completely inactive when grown in glucose or when both glucose and galactose are fermented together (180). On the other hand, in the presence of galactose, fermentation during growth may preserve the above required factors due to their incomplete utilization, and thus present a condition accounting for observed "adaptations."

**Effect of Oxygen on "Synthesis of Galactozymase" and Abolition of Induction Periods.**—Using nondividing yeast cells (suspensions in phosphate buffer), Spiegelman (172) described two types of yeast strains: one capable of fermenting galactose under anaerobic conditions, and one requiring a pretreatment with oxygen. The rate of anaerobic "adaptation" was approximately one-fortieth of that in oxygen. The nondividing yeast with which Stephenson and Yudkin (181) experimented exercised galactozymase activity under both aerobic and anaerobic conditions. Many unadapted yeasts have shown the ability to ferment galactose after a sufficient induction period. In an atmosphere of nitrogen, the induction period extended indefinitely, whereas pretreatment with oxygen materially shortened it (150). Spiegelman (172) stated that those strains unable to adapt themselves to galactose anaerobically fermented it under this condition only when an adequate enzyme activity had been built up by oxygen pretreatment.

It must be pointed out that the production of an adaptive enzyme for galactose has been claimed to occur in aqueous solutions in the absence of multiplication, and not as a result of the selection of individual cells from a population (172, 181). Thus, we are dealing with chemical reactions which bring out the *already existing* ability of the yeast to ferment galactose, which for reasons to be discussed further, would seem to require various periods of induction, involving the formation of critical substrates. Before an objective analysis of the experimental data relating to this question is made, other hypothetical possibilities may be considered. The fact is that contact of the cells with a substrate under anaerobic conditions does not bring out the activity of the enzyme system for a long time. In contrast, the activity is demonstrable within one hour or so after the cells are treated with oxygen. These facts may occasion certain speculations. *The appearance of activity does not appear to be due to an effect of the substrate whereby certain cell proteins undergo a configurational change with the acquisition of enzymic activity specific toward the substrate.* For, if this were the case,

this effect would be the same under both anaerobic and aerobic conditions, unless by oxygen treatment the protein molecule is oxidized, yielding the so-called adaptive enzyme. However, in view of the experiences of Stephenson and Yudkin (181) who showed that enzyme activity, under the experimental conditions they used, was independent of aerobic factors, a configurational change in a cell protein, yielding a "new enzyme," is improbable.

The shortening of the galactose fermentation induction period by oxygen treatment may also be interpreted in the following way. Oxygen treatment may cause aerobic glycolysis of the yeast polysaccharides. The fermentation of galactose, unlike that of glucose, may not take place until the polysaccharides undergo oxidative glycolysis whereby they are removed as barriers against galactose, which then can get at the key enzymes. This idea may be worthy of consideration because of the fact that the endogenous aerobic respiration of yeast cells is an active process, and that the appearance of galactose-fermenting ability, in some cases, appears to follow it.

Despite these hypothetical possibilities, we are inclined to believe that the following discussion provides the most plausible answer to the question.

**Possible Formation of Critical Hexose Diphosphate by Oxygen Treatment.**—Stephenson *et al.* (179, 181) state that galactose-adapted yeast loses its galactozymase after fermentation of glucose, or when glucose is added to the galactose-fermenting mixture. Apparently, glucose fermentation robs the cell of a critical factor which cannot readily be synthesized from galactose when its fermentation succeeds that of glucose. This critical factor is produced very slowly (24 hours) by the cell under anaerobic conditions and within an hour or two in the presence of oxygen; it is produced from a cell material which most probably is yeast glycogen or polysaccharide. The fact that Cattaneo (18) had to use acetaldehyde and hexose diphosphate to cause fermentation of galactose, despite the fact that he used a sample of yeast which had been grown in galactose-containing medium, emphasizes the critical nature of the factors found under one set of conditions, and their loss or exhaustion under another set of experimental conditions.

The production of critical factors, required for the fermentation of galactose more than for that of glucose, appears to take place most readily when the cells are pretreated with oxygen, as discussed above. Spiegelman and Nozawa (174), measuring the rate of endogenous respiration or the aerobic dissimilation of reserve carbohydrate, found that the respiratory quotient (R.Q.) was unity or near this value. The washed suspensions of

yeast, after 24 hours under anaerobic incubation, did not show a decrease in oxygen uptake. After an anaerobic period there was however, a temporary burst of activity, which rarely exceeded 50 minutes. During this activity, the rate of oxygen consumption was in excess by almost 40%. The R.Q. during this time was close to unity. In contradiction to the conclusion of these investigators, the burst of activity following anaerobiosis appears to have been due to an accumulation of a catalytic amount of hexose diphosphate or hexose monophosphate from the hydrolysis of yeast glycogen by yeast phosphorylase. This amount of hexose phosphate was insufficient to change the R.Q. values of endogenous respiration but sufficient to cause the observed burst of activity, a phenomenon comparable to the elimination of induction periods or to the acceleration of slowly progressing fermentation by the addition of hexosediphosphate. This interpretation is supported by the observations of Cori (25) on animal tissue. According to Cori:

"The increased oxygen consumption which sets in following anaerobic muscular contraction reverses the glycolytic mechanism and leads to the resynthesis of glycogen from lactate and from hexose monophosphate, both of which accumulate during the anaerobic phase."

It seems reasonable to conclude that, with the formation of phosphorylated hexoses, the increased ability of the yeast to ferment galactose becomes evident.

These statements may clarify, or offer an experimental explanation for, certain discrepancies observed during the fermentation of galactose by various cell systems. We are dealing with a reaction mechanism rather than with the synthesis of a new enzyme, adaptive or otherwise. We are dealing with frequent or constant changes which affect the amounts and activities of critical factors. These seem to affect all four hexoses qualitatively in an identical manner, and quantitatively to varying degrees.

The above considerations apply also to the "adaptive" nature of the galactozymase of *Escherichia coli* (178) and of *Streptococcus lactis* (136). For, experiments by Hegarty (62) with the latter organism show definitely that galactozymase activity is an inherent property of the cell and is not produced adaptively. Also, *E. coli* grown on glucose or plain broth, exercises a definite galactose-fermenting ability. This ability probably is greater when galactose is present in the medium. Hegarty (62) found that *S. lactis* grown in glucose-tryptone broth is also able actively to attack galactose, lactose, sucrose, and maltose when the fermenting cells are harvested from the logarithmic phase of growth, but attacks these sugars

only with difficulty, if at all, when the cells are aged. The cells are stripped of critical fermentation factors during aging.\*

**Hexokinase or Galactozymase?**—In the fermentation of galactose, the important enzyme is hexokinase, for it transforms galactose into glucose-6-phosphate or fructose diphosphate, and from there on the difference between galactose and other hexoses completely disappears. "Galactozymase" is a name inherited from a period when the chemical mechanism of fermentation reactions was less clearly understood. Whether there is a specific galactose hexokinase is the question. From the previously described experimental data, it seems that heterophosphatase or hexokinase does not differentiate between the four hexoses. Highly purified hexokinase converts both fructose and glucose (perhaps also mannose and galactose) into glucose phosphates. For the fermentation of galactose by yeast (presumably also by other cells), whether adapted or not, phosphorylated hexoses seem to be the most critical substances. The roles of adenylic acid and other phosphorylated compounds need to be studied in this respect. The importance of hexose diphosphate, not only as catalyst in the initial stages of fermentation, but also as antagonist to the specific inhibitor of hexokinase, glyceraldehyde, requires emphasis.

**Reversible Inhibition of Hexokinase by Glyceraldehyde.**—Stickland (182) reported that *dl*-glyceraldehyde inhibits glucolysis (fermentation), but has no effect on the rate of glycolysis of starch. Glyceraldehyde (0.003 *M*) causes almost complete inhibition of the fermentation of both glucose and fructose. This inhibition has been shown to be specifically toward hexokinase; it is reversed by excess hexokinase, for then there is enough free hexokinase to still effect the phosphorylation of glucose or fructose. Evidently the glyceraldehyde-hexokinase complex is fairly stable and weakly dissociable. Similarly, 0.0004 *M* pyruvate abolishes the inhibition by an 8-fold greater amount of glyceraldehyde. According to Parnas (130), the inhibition of glucose fermentation by glyceraldehyde is reversed by fructose-1,6-diphosphate. Since the latter, on splitting, yields dioxyacetone phosphate and phosphoglyceraldehyde, it is interesting to note that one of the fermentation products inhibits the fermentation process at its earliest step by inhibiting hexokinase. The reversal of this inhibition by pyruvate and fructose-1,6-phosphate may not be due to a competitive reaction. It may be that pyruvate, as phosphopyruvate, and fructose diphosphate phosphorylate glyceraldehyde and thereby eliminate it by transforming it into readily oxidizable form as indicated by reaction (1) on page

\* Hegarty, contrary to his own experimental findings, considers the ability of physiologically youthful organisms to ferment galactose to be a rapid adaptation.



73. The abolition of the induction period or the appearance of galactose-fermenting ability after oxygen treatment of yeast cells may also be interpreted in the light of these reactions. That yeasts or other cells grown in various media contain glyceraldehyde combined with cell hexokinase is a possibility which must not be overlooked.

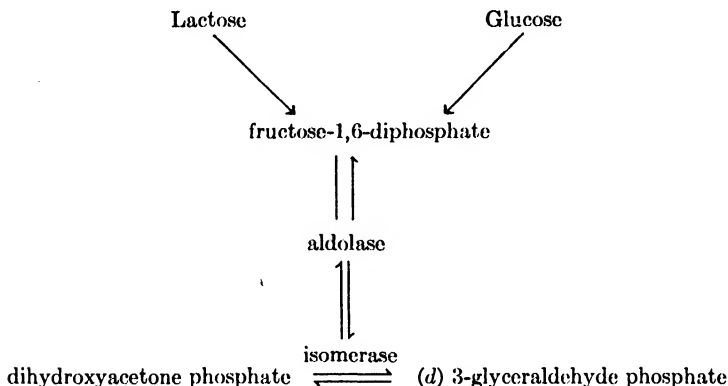
### 2. *Is Melibiose Zymase an Adaptive Enzyme?*

It has recently been reported (97, 173) that a strain of yeast, *Saccharomyces carlsbergensis*, grown for 48 hours in a medium containing yeast extract, bactopectone, salts, sodium lactate and glucose (Cerelease), washed twice with 0.066 *M* potassium dihydrogen phosphate solution and then suspended in this phosphate did not ferment melibiose after incubation for eight hours in an atmosphere of nitrogen. However, when similarly prepared suspensions of yeast were incubated in air for 100 minutes in the presence of melibiose, fermentation, as evidenced by carbon dioxide evolution, took place. The statement was made that, under these nonproliferating conditions, the gene initiates the synthesis of melibiose fermenting enzyme complex, providing melibiose is present. These results may be interpreted on the basis of our previous discussion. The aerobic incubation for 100 minutes corresponds to the induction period and is necessary for the synthesis of phosphorylated sugars in catalytic amounts, phosphorylation involving aerobic oxidation-reduction reactions. Melibiose is a glucosidic galactoside. The fact that aerobic conditions are necessary for its fermentation shows that the "adaptation" does not involve the synthesis of a glucosidase to bring about the hydrolysis of the disaccharide. In this case we are dealing with the fermentation of galactose as well as glucose, obtained upon hydrolysis of melibiose; the fermentation apparently required a period for the synthesis of phosphorylated hexoses and not a special melibiose zymase.

### 3. *Is Dihydroxyacetone Zymase an Adaptive Enzyme?*

Karström (75) reported that the fermentation of dihydroxyacetone by *Escherichia coli* begins promptly. When the organism was grown in glucose or lactose, the fermentation of dihydroxyacetone by washed cells required an induction period of 10 hours, and at the end of 60 hours the total fermentation was at its maximum or was equal to that occurring when the cells were grown in dihydroxyacetone. Karström attributed this difference to the synthesis of an "adaptive" enzyme specific for dihydroxyacetone. Since the cells grown in glucose or lactose fermented dihydroxyacetone in an

environment which did not permit multiplication, the induction period must be attributed to the synthesis of critical factors lost after growth. Furthermore, since the fermentation of glucose or lactose involves or leads to the fermentation also of dihydroxyacetone, there appears no reason for believing that there is a special, adaptive fermentation enzyme for dihydroxyacetone.



In the fermentation of dihydroxyacetone, either the enzyme which phosphorylates dihydroxyacetone, or the conditions controlling the activity of isomerase in shifting the equilibrium from left to right, can at the most be considered as critical factors for the above described induction periods. *E. coli*, adapted or not, ferments glucose all the way to pyruvate, acetate, and formate. The above two factors must therefore be the integral components of the cells. We must therefore look, not for a new enzyme, but for the conditions which are deficient or antagonistic for the explanation of these discrepancies.

#### 4. *Is Formic Hydrogenlyase an Adaptive Enzyme?*

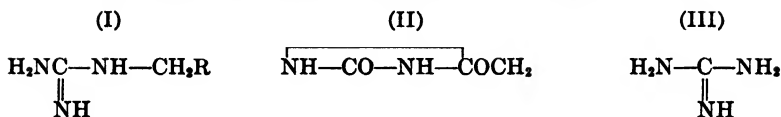
Formic hydrogenlyase catalyses the reaction:  $\text{HCOOH} \rightleftharpoons \text{CO}_2 + \text{H}_2$ . It is difficult to see how any one of the reactants in this equilibrium could be instrumental in the synthesis of a specific enzyme. None of the reactants is requisite for the synthesis of an enzyme, unless the presence of formate exercises a definite influence on the kinetics of the reaction, shifting the equilibrium toward the right. Despite the fact that *E. coli* was grown in sodium formate, it required 60 minutes before the evolution of hydrogen was evident (178). The nonadapted cells exercised, however, a definite activity, though on a smaller scale. This, in our opinion, was due to an imperfect metabolic environment in which the cells were being tested.

our experiments (157), using washed suspensions of *E. coli* grown in glucose broth, it was observed that in the presence of 0.05% yeast extract, in comparison to the reaction in salt-buffer mixture, the evolution of hydrogen increased more than fourfold. Since under these conditions there was no multiplication, this increase of hydrogen evolution was attributed to certain coenzyme factors present in the yeast extract. In addition, during the oxidation of glucose by *E. coli*, the activity of hydrogenlyase was dependent on various factors. In salt-buffer medium, or trace of yeast extract, or 0.18% neopeptone, the volume of oxygen consumed was greater than that of hydrogen evolved. On suppressing the oxygen uptake by sulfanilamide, a greater volume of hydrogen was evolved. In serum and yeast extract, the volume of oxygen consumed was equal to that of the hydrogen evolved. On suppressing the oxygen uptake by sulfanilamide or *p*-aminobenzoic acid, the volume of hydrogen evolved increase several fold. These facts show that *E. coli* grown in glucose contains hydrogenlyase in a very active form and does not require adaptation by growing in the presence of formic acid.

The reversible decomposition of formic acid is only a part of a complex reaction equilibrium (99, 196). Depending on the environmental conditions, formic acid reversibly reacts with acetyl phosphate, yielding pyruvate and inorganic phosphate. This is dependent on the presence of an excess of adenylyl pyrophosphate and involves an active phosphorylation reaction. In the absence of such factors, the cells may manifest a greater activity in decomposing formic acid. Interesting studies are being carried out by Lipmann and co-workers (99) in this field.

### 5. *Is Creatinine Decomposition an Adaptive Process?*

Dubos and Miller (36) and Dubos (35c) reported that certain strains of bacteria isolated from soil adapted themselves to the decomposition of creatinine when grown in its presence. These workers state that the production of a specific enzyme is favored by the presence of creatinine (or creatine) in the absence of any cellular multiplication. The "adapted" enzyme was capable also of decomposing creatine derivatives, such as creatinine, guanidineacetic acid, methylguanidine, arginine, etc., all of which possess the common guanidine group. In contrast, it was unable to decompose methylhydantoin, methylhydantoic acid, hydantoin, sarcosine, or guanidine. That is, the enzyme was capable of decomposing substances corresponding to the general formula I but was incapable of acting on hydantoin (II), or of converting guanidine (III) into urea. The principal



product of the substances decomposed was urea, practically in theoretical amounts. That is, the enzyme which was present in the normal cells was specific for the active group of the substrates rather than for a single substance.

Now, the hydrolysis of arginine by arginase involves the addition of one molecule of water to arginine yielding two hydrolytic products, namely, urea and ornithine. The fact that the cells in the above experiments decomposed arginine, yielding urea, in the same way that they decomposed creatine and related substances to yield urea indicates that the "adaptive" enzyme is either identical or similar to arginase.

The fact that cells grown in creatinine show an accelerated activity toward its decomposition may mean that cells saturated with creatinine can more readily decompose it. This may merely be a matter of reaction kinetics rather than a new or increased synthesis of an enzyme, and may be particularly true because of the fact that the cells were autolyzed readily, losing their enzymes. In the presence of excess substrate, the formation of enzyme-substrate complex would favor the protection of the enzyme.

#### 6. Relation of Autolytic Processes to Cell Activities

In many cases, weak enzyme activities are due to the loss by cells of a specific enzyme or of enzymes whose activities control, directly or indirectly, other enzymes. Serious errors or faulty interpretations may be made when the state of the cell has not been a concern of the investigators. Reference has already been made to the finding of Hegarty that cells harvested during their physiological youth are more complete in their enzyme activities than aged ones.

In our experiments with *Staphylococcus aureus* (163), it was observed that when the organisms were grown in media containing glucose, the harvested washed cells were completely devoid of the ability to dismute pyruvic acid. In contrast, when they were grown in the absence of glucose, in plain broth or casein hydrolyzate, they were highly active in this respect. An extensive later study (unpublished) with E. A. Swart showed that washed cells grown in glucose, harvested after a growth period of 8 hours, were relatively more active than those grown for a longer period. The activity declined as growth proceeded. After a period of 24 hours, the cells were completely inactive. All of the glucose was dissimilated during this period of growth. The addition of cocarboxylase (or thiamin) did not restore the activity. There is no doubt that carboxylase and other activities are lost during growth in glucose.

Another example is the loss of enzymes by certain strains of butyric acid bacteria (152). Investigating the loss of various enzymes and the loss of Gram-staining property at various periods of growth, it was found that there was an autolytic factor in the growth fluid which dissolved out the factors responsible for the Gram-positive staining property of the cell. Simultaneously with the loss of this property, there occurred the loss of cytochrome components and the ability to oxidize ethyl alcohol and glucose.

In a similar study with *Pneumococcus* (153), it was shown that with the loss of Gram-positive staining property the activity of the respiratory enzymes was lost. A subsequent extensive study by Dubos (35) showed that *Pneumococcus*, similar to butyric acid bacteria, released an autolytic enzyme which caused the loss of Gram-positive staining property.

Since protein-ribonucleate complex has been found to be involved in Gram-positive staining (4, 63, 64), these losses of enzyme activities must also be associated with the loss of protein-ribonucleate complex.

Reference has already been made to the observation of Dubos and Miller that creatinine-hydrolyzing bacteria tend to become autolyzed, losing their enzyme activities. In studies with Gram-negative microorganisms, it is not possible, however, to differentiate, by staining technique, the damaged or partially autolyzed cells from healthy ones. Under these circumstances, a study of the activities of various enzymes at various stages of growth and under various environmental conditions is the best way to detect the changes the cells may undergo. This precaution is necessary to avoid speculative and erroneous conclusions.

### 7. *Is Reversal of Inactivation an Adaptive Process?*

Bonner, Tatum, and Beadle (9) reported that an x-ray mutant strain of *Neurospora* which required isoleucine and valine adapted itself in time to grow on media containing neither, or, at most, suboptimal concentrations of these amino acids. After becoming so adapted, the cells grew at the rate characteristic of their normal growth.

Stokes, Foster, and Woodward (183) found that an x-ray mutant strain of *Neurospora sitophila* which required pyridoxine for growth could synthesize pyridoxine under certain conditions of nitrogen nutrition at a rate necessary for normal growth if the pH remained above 5.8. Ammonium compounds served as nitrogen source for the synthesis of pyridoxine. Nitrate, nitrite, amino, amide, and other forms of nitrogen were inadequate for this mold in this respect.

A mutant strain of *Neurospora* requiring *p*-aminobenzoic acid likewise regained the ability to grow in the absence of this substance after an incubation period of two or three weeks (213). In this case, a pH effect on the regeneration of the ability to grow in the absence of *p*-aminobenzoic acid was not observed.

Bonner, Tatum, and Beadle (9) are of the opinion that these mutant strains were capable of making these substances by some alternative mechanism, thereby circumventing genetic block. It must be noted, however, that these workers see "a difficulty of interpretation as to the

point of block since it is at least theoretically possible that any substance which brings about a growth does so not directly, supplying the needed product of the blocked reaction, but rather indirectly by *accelerating adaptation*." They distinguished between this adaptation and the normal growth response as the difference in the length of time required to attain a normal growth rate.

As we see it, the restoration of certain abilities after a period of time may have been due, not to the above postulated adaptive process, but to the time or the chemical or physicochemical environment which caused the reversal to the native state of certain enzyme proteins which might have been deformed, distorted, or denatured under the effect of x-ray treatment. Proteins are denatured by ultraviolet or cathode ray treatment (128). Under the influence of irradiation, proteins undergo denaturation and a photochemical reaction, followed by flocculation of the denatured protein. Alteration of the state of aggregation, causing biological inactivation and shift of *pH* of solutions toward the isoelectric point of the protein, is of frequent occurrence.

At least in one case a change of *pH* to the alkaline side reversed the inactivation observed with the pyridoxine-less *Neurospora* x-ray mutant strain. The literature on the reversal of denatured enzyme proteins has been critically reviewed by Neurath *et al.* (128). Depending on the condition and the extent of denaturation, this process has been found to be reversible, with the restoration of biological activity. It is therefore plausible that the above cited reactivations in x-ray mutant *Neurospora* strains are reversals of denatured systems rather than "adaptive" processes.

The reactivated or the inactivated enzyme proteins are inheritable. This raises the question of whether the reactivations of these systems are associated with the reversal of the mutant genes to the normal state. In other words, is the reactivation directly controlled by the respective genes? For it is stated by Tatum (187) that the general concept of gene action is that not only biochemical or metabolic defects are gene controlled, but that genes are the fundamental controlling factors of all biochemical reactions in an organism.

A personal communication from Tatum, however, states: "Strain 16117 typically adapts as do a number of other strains requiring other substances without a change in the mutant genes. Many other strains apparently will not adapt under any conditions so far tried. There seems to be no particular relationship between the substances required and the ability to undergo adaptive changes. In a few instances, adaptation may involve gene reversions." On the basis of these observations, it would seem that, in these cases also, so-called "adaptation" does not occur in response to the direct stimulating action of the substrate, but in response to a chemical environment. Tatum's

remarks are explainable by the reversible and irreversible denaturation of the enzyme systems.

The fact that restoration of the activity of the x-ray treated systems occurs without concomitant restoration of the mutant genes to the original state suggests that the two may occur simultaneously but are independent of each other, or independent of the control of the enzyme systems, in this respect, by the specific gene. May it not be that the gene controls the synthesis and determines the species specificity of the enzyme complex without being responsible for the chemical activities of the enzymes, or for their inactivation and reactivation reactions? For, how could we explain numerous processes whereby the enzymes of a cell are damaged, or removed from the cell without affecting the genes, as shown by the regeneration of the complete enzyme system of the original cell when a new generation is produced? We ask these questions in view of the fact that these relationships may have a bearing on the mechanism of the development of resistance, its inheritance, its reversal, etc.

#### 8. *Theoretical Considerations*

The available data do not support the existence of "adaptive" enzymes. In all the cases considered, the enzymes were present as integral parts of the cells, but defects in the metabolic environment greatly reduced their activities. Under favorable conditions, their activities were manifest. What these favorable conditions are must be individually investigated to determine the missing adjuvant factors. In the case of galactose-fermenting systems, hexose diphosphate, with or without adenylic acid or adenosine triphosphate, seems to play a critical role. In the presence of a large amount of specific substrates, the necessary factors seem to be either protected or prevented from becoming exhausted. This may arise from a difference in the rate and extent of fermentation of one or the other hexoses.

Another point which requires consideration is that a substrate which is specific for the "adaptive" enzyme is invariably a very close configurational relative of the class of substrates which are readily metabolized by the cell, such as galactose, glucose, mannose, fructose, etc., or arginine, creatine, etc. All these hexose substrates are metabolized in the same manner after they have been phosphorylated to glucose-6-phosphate. Except for the first step in the phosphorylation reaction, galactose is not a special case, different from glucose. Dihydroxyacetone does not differ from that produced during the fermentation of the four hexoses. It is either reduced to glycerin or oxidized to pyruvic acid. We have already pointed out the similarity between creatinine and arginine, which possess

identical hydrolyzable groups upon which a single hydrolytic enzyme can act to yield urea. Similarly, Anson's crystalline carboxypeptidase hydrolyzes numerous dipeptides composed of different amino acids. There is no doubt that, for configurational reasons, the rate of the hydrolysis of one dipeptide will be different from another; even the rate of one member of the class of dipeptides may be immeasurably small. Similarly, pyruvic, methyl, and dimethyl pyruvic acids are decarboxylated by the same carboxylase (127). A single  $\beta$ -glucosidase will hydrolyze at extremely different rates numerous  $\beta$ -glycosides, irrespective of the nature of the glycosidic groups (132b). Tyrosinase will oxidize mono- and polyphenols, tyrosine, and even certain of the sex hormones (126), also phenols or catechols possessing a long unsaturated side chain attached to the aromatic ring (167).

Göpfert and Nord (48b) found that methyl alcohol, previously considered unattackable, is dehydrogenated to formaldehyde without adaptation when methyl alcohol is added to glucose-containing medium one week after inoculation with *Fusaria*. *Fusaria* thus grown can dehydrogenate methyl alcohol because the organisms contain an alcohol dehydrogenase capable of dehydrogenating it, as well as higher primary (and secondary) alcohols. However, due to the difference in the rates of dehydrogenation of various alcohols, the ability of the organism to perform this reaction with one or the other alcohol is demonstrable under the same conditions only when an appropriate mass of organisms is made use of as the source of enzyme.

We conclude, therefore, that cells do not synthesize new, "adaptive" enzymes which attack substrates lacking in reactive groups present in substrates which are normally readily metabolized by the cell. *Staphylococcus aureus*, for example, does not synthesize an adaptive hydrogenlyase if it lacks a pre-existing enzyme system capable of functioning as hydrogenlyase. An organism is incapable of learning to metabolize type specific pneumococcal carbohydrates, for example, unless its own carbohydrates contain the same enzymatically reactive groups. Numerous examples can be cited to support this statement. Pneumococcal cells do not contain catalase, but contain certain heme-containing enzyme systems. Despite the fact that this organism may have been grown in blood or in purified catalase solutions for many generations, it still is incapable of making use of these environmental conditions to synthesize catalase. *Hemophilus influenzae*, requiring cozymase I and heme for growth, is incapable of synthesizing enzymes to produce these factors, even though it may have been subjected to their "stimulating" influence for many generations.



An often-cited example of adaptation is the claimed ability of *Eberthella typhosa* to synthesize tryptophan (45) by gradual decrease in the tryptophan concentration of the medium. The assumption is that the diminishing concentration forces the organism to synthesize its own tryptophan. But another interpretation is possible. The fact is that a given bacterial culture contains numerous variants with varying biochemical activities, those capable of synthesizing tryptophan and those which are incapable of doing so. By decreasing the concentration of tryptophan those variants which are incapable of synthesizing this amino acid are starved out, leaving in the medium those which are nonexacting (81, 92). Such an explanation may also be applied to the observation by Silverman and Werkman (166) that *Propionibacterium pentosaceum* acquires the ability to synthesize vitamin B<sub>1</sub>. In our own experience (155), starting with a given culture of *Staphylococcus aureus*, we derived three variants with respect to tryptophan requirement: *nonexacting*, *partially exacting*, and *exacting*. The exacting strain was incapable of synthesizing tryptophan unless pantothenate and glucose were present in the medium. This variant, after a year's experimentation, was still incapable of synthesizing tryptophan (or pantothenate), despite the fact that it synthesized its own tryptophan through the agency of pantothenate.

In order that a cell acquire the ability to synthesize new enzymes and also be able to transmit this ability to successive generations the evolution of a higher genetic type is necessary. This is the subject of the following section.

## V. Building-Up Species Characteristics by Genetic Factors

### 1. Transformation in *Pneumococcal* Types

To the students of the *Pneumococcus* the findings of Griffith (53) regarding the transformation of one type of *Pneumococcus* into another is a familiar subject. Griffith achieved such transformation by injecting into an animal a nonvirulent *Pneumococcus* (derived from type II) and a heat-killed type III *Pneumococcus*. The former acquired the characteristics of the living form of the latter. (A review of subsequent studies with *Pneumococcus* and virus will be found in White's monograph, 206, and in a paper by Avery *et al.*, 2.)

The change from virulent *Pneumococcus* type II into noncapsulated, non-virulent form also occurs when the former is successively cultivated in the presence of type II antipneumococcal rabbit serum. This may require as many as 36 serial passages. The resulting *Pneumococcus* loses all of the

specific characteristics of type II. Conversely, a nonvirulent *Pneumococcus*, cultivated in an homologous immune serum, or passed through an animal, acquires the original type specific characteristics. While the former is a degradative, hereditary change, in the latter case a building-up process occurs.

Up to this point, the serological type specificities of various pneumococcal cells have been characterized by their respective cellular polysaccharides without definite information regarding the nature of the agent which brings about the above-mentioned and similar changes. Avery *et al.* (2) have recently reported the results of a study which, from the standpoint of genetic variations and of the processes responsible for the building up of species characteristics, is of fundamental importance. They found that the factor which transforms the nontype specific, nonvirulent to type specific, capsulated *Pneumococcus* may be desoxyribose nucleic acid. A 0.003  $\mu$ g. quantity of this material per 2.25 ml., or one part in about  $6 \times 10^8$  parts of an appropriate serum broth medium, is sufficient to bring about this transformation. The type specificity or newly transformed *Pneumococcus* is determined by the type specificity of the cocci from which this agent has been obtained. Types I, II, III, IV, and XIV have thus been transformed.

The desoxyribose nucleic acid (from type III cells), besides the usual qualitative and quantitative chemical properties, is reported to have a molecular weight of about 500,000 and to be homogeneous electrophoretically. It is stable at 65° C. for 30 minutes; the highly purified samples are less stable. The presence of pneumococcal protein and carbohydrate could not be demonstrated serologically, though a faint trace reaction in precipitin tests with potent type III pneumococcal rabbit sera was present. Crystalline trypsin or chymotrypsin or a combination of both, and crystalline *d*-ribonuclease had no effect on this nucleic acid. Its activity was destroyed by the action of desoxyribose-nucleodepolymerase.

According to McCarty (107), ascorbic acid, quinones, and quinone-yielding compounds inactivate desoxyribose nucleic acid in the presence of oxygen. This inactivation is counteracted by sulfhydryl compounds. The inactivation does not alter the physical and chemical properties of the agent. In the light of McCarty's findings, it might be suggested that the molecule contains a reactive sulfhydryl group. McCarty discusses this possibility but does not accept it, though no tests for the —SH group are mentioned.

It is interesting that attempts to induce transformations in suspensions of resting cells thus far have proved unsuccessful. No support, then, is given from this source to the possibility that the enzymes involved in the

transformation are "adaptive." Transformation occurring during multiplication in the first transplant is thereafter transmitted to successive generations without any further addition of the transforming agent.

The principal characteristics of a type specific *Pneumococcus* is its ability to synthesize this transforming agent and the type specific capsular polysaccharide. While the type specific polysaccharides are serological indicators, the transforming agent appears to be the most nearly associated with the hereditary factors of the more than fifty pneumococcal types. This means that the nucleic acids must occur in so many configurational forms to account for all these types. And if, in all the species, the nucleic acid is the sole determinant factor, there must be as many nucleic acid configurations as there are types or species.

What subtle configurational differences could be present among nucleic acids which do not show immunizing activity and cannot, therefore, be differentiated by serological procedures which are capable of detecting differences between substances containing *d*- and *l*-active antigenic components? Could it be that the transforming agent still contains a highly potent type specific factor which, in amounts very much less than 0.003  $\mu$ g. per sec. 2.25 ml. is responsible for this effect?

According to our present state of knowledge of the structure of nucleic acid, it is a polymer of tetranucleotides, which does not appear to offer the possibility of many stereochemical variations. Its polymer state may perhaps be compared to glycogen or starch, in which there are only  $\alpha$ -1,6- and  $\alpha$ -1,4-glucosidic linkages made up of glucose molecules. On hydrolysis by enzymes, these polymers yield principally *d*-glucose, maltose, and di- and tetrasaccharides of *d*-glucose (26, 118). While the transforming agent sponsors complete change of type and likewise its own synthesis, glycogen or starch is responsible for the priming of the synthesis of similar polysaccharides. According to Cori and Cori (27) and Cori (26), no polysaccharide synthesis will take place with purified phosphorylase, unless a small amount of polysaccharide is present in the system. The type of phosphorylase used and not that of the activating polysaccharide determines the nature of the polysaccharide synthesized. For example, muscle phosphorylase, when primed with liver glycogen, synthesizes a typical starch *in vitro*, and liver phosphorylase, when primed with plant starch, synthesizes glycogen. Muscle phosphorylase also synthesizes glycogen in the intact cell. Is it possible that nucleic acids are exercising similar effects in pneumococcal transformations? McCarty and Avery (107a) are not inclined to accept the possible presence of a specific protein in their preparation as the seat of transforming activity. They see, however, the necessity for demonstrating

configurational and structural differences among desoxyribosenucleic acids for a final proof.

## 2. Genetic Observations on *Paramecia*

Sonneborn (171) describes four races of *Paramecium aurelia*, of which one was a "killer," and the other three, "sensitive" races. The killing of sensitive animals was preceded by characteristic morphological aberrations, particularly by a shifting of the posterior part of the body to the aboral side. It was possible to cross killers with sensitives. During mating, there was no injury to the sensitive member of the pair. One of the two exconjugants of each pair was a killer and the other, a sensitive. The killers were those that derived cytoplasm from the killer parent, while the sensitives derived cytoplasm from the sensitive parent. The character, "killer," was dependent upon a cytoplasmic substance which normally failed to accompany the "male" gamete nucleus as it passed from one mate to the other during conjugation. The cytoplasmic differences could be perpetuated, not by independent cytoplasmic determinants, but by genes which were alike in the two forms crossed; and these genes, like the killer gene, may be unable to initiate production of the cytoplasmic substances involved. Only by means of specially favorable material can the genic control of the cytoplasmic substances be discovered.

By transposition of cytoplasm from a killer to a sensitive cell through a cytoplasmic connecting bridge, the latter was transformed into a killer. The new character was inherited in all subsequent generations by those progeny that obtained their cytoplasm from the transformed individuals.

Sonneborn proposed a hypothesis to explain his observations, which we will not go into. Discussing the transformations of *Pneumococcus* he offers a few examples of how the cells learn to synthesize tryptophan, vitamin B<sub>1</sub>, etc. As discussed previously, the experimental basis of the examples cited is invalid. This aspect of his interpretations requires revision. However, his statement that the same gene may be involved in all fifty types of *Pneumococcus*, and that there are as many cytoplasmic materials which the gene can act upon to produce the respective types, may represent a point of interest in connection with the observations of Avery *et al.* (2), that is, if Sonneborn's "cytoplasmic materials" are contributory as genetic factors, and not suggestive of the "adaptive" enzyme concept. For it must be remembered that Avery's purified material appears to act as a catalyst of genetic caliber and not as a substrate.

Studying antigenic variants in cultures of paramecia, Harrison and Fowler (60) determined the antigenic changes in cultures of *P. bursaria*

following the mating phenomenon. Using two antigenically distinct strains, one colored brilliantly green by zoochorellae inclusions, the other colorless, they observed that about 95% of all recent (within one hour) exconjugants contained some antigenic substance common to their mates which was not previously present within themselves. The new antigenic character of the exconjugants persisted in their progeny for at least one month of active growth, or for sixty generations or more. These investigators are inclined to believe that the antigens involved in the reaction are very largely, if not exclusively, cytoplasmic in character, and that cytoplasm is interchanged during the course of conjugation in these animals.

### 3. Acquisition of Vitamin-Synthesizing Abilities by Mating Different Species of Yeast

Lindegren and Lindegren (98) studied the effect of mating two species of yeast on the vitamin-synthesizing ability of the hybrid. One species, *Saccharomyces carlsbergensis*, was capable of synthesizing both biotin and pantothenic acid in large quantities, but was unable to synthesize pyridoxine. The other, *S. cerevisiae*, was deficient in the ability to synthesize biotin, and varied from good to poor in the ability to synthesize pantothenic acid. The hybrid was capable of synthesizing all three vitamins. Two other species of yeast were mated, with similar results. The species, *S. globulus*, which was incapable of synthesizing thiamin but capable of synthesizing pantothenic acid was mated with a pantothenic acid-deficient species of *S. cerevisiae* (a different strain from the one used above). The hybrid was capable of synthesizing both pantothenic acid and thiamin. The hybrid was a poor synthesizer of biotin, since neither parents possessed this ability. The acquired abilities of the hybrids were attributed to the genes inherited from the parent species.

### 4. Abolition of Resistance of Trypanosomes by Fertilization

As will be discussed in the succeeding section, orthoquinoid dyes deprive trypanosomes of blepharoplast (kinetocore). This is one of the changes associated with the development of resistance to the causative toxic agent. Gonder (49) passed *Trypanosoma lewisi*, rendered resistant against arsenophenylglycine, first through a rat louse, *Haematopinus spinulosus*, and then to a healthy rat. As a consequence, the trypanosomes lost their resistance and became highly susceptible to the drug. Care was taken to see that the trypanosomes performed "an act of mating in the rat louse," thus establishing the fact that the "fertilization" and the development of the

trypanosomes in this host brought about this reversion. Evidently, a genetic and biochemical mutation had taken place in the fertilized trypanosomes. A similar experiment by Gonder with *Spirochaeta recurrentis* and *S. gallinarum*, resistant to salvarsan, yielded negative results, since these organisms, like bacteria, multiply without sexual fertilization. The significant fact is that arsenic-fast trypanosomal strains can lose their resistance to, or regain their susceptibility to, drugs by simple passage through a rat louse.

### 5. Conclusion

The cited experimental observations show that an increase in the synthesizing ability of a living cell can occur only by a building-up process. This requires the acquisition by the cell of genetic factors from other cells, and the ability to transmit the acquired factors to succeeding generations.

## VI. Degenerative Mutations and Resistance to Inhibitors

### 1. Relation of Degradative Mutations to Resistance

**Terms "Adaptation" and "Resistance."**—In a preceding section it was shown that claims for the production or synthesis of "adaptive" enzymes have been based on inadequate study of the metabolic factors of the respective systems. On the other hand, we discussed various studies showing a definite increase in the enzyme content of cells under certain conditions. With *Pneumococcus* and yeast cells, it has been shown that higher genetic types are evolved, and that an increase in the kind and contents of enzymes is attained by incorporating genetic factors into the cellular structure. These building-up processes do not constitute examples of the synthesis or production of "adaptive" enzymes.

The term "adaptation" has, in the past, been used by biologists to indicate a readjustment to changed environmental conditions without any information regarding the biochemical factors involved in this adjustment. Since the terms "adaptation" and "adaptive enzymes" are indicative or suggestive of the same concept, and since there is no experimental basis for either, it is suggested by the present writer that these terms be dropped, and that terms be used which can be experimentally defined. There appears to be a biochemical basis for the term "resistance" or for "development of resistance" to metabolic inhibitors. The development of resistance seems to be related to degradative genetic mutations occurring under the influence of metabolic inhibitors.

**Mutations Caused by X-Ray and Ultraviolet.**—Beadle, Tatum, and associates, subjecting *Neurospora crassa* to x-ray radiation, obtained biochemically deficient mutants. Approximately 60,000 single spore cultures were tested. Of these, approximately 400 had undergone biochemical mutations. At least 40 of the latter group were identified as requiring specific, single organic compounds for growth. In general these mutations involved only a single gene.

Distinct mutant strains of *Neurospora* have been isolated which require each of the B vitamins except riboflavin and folic acid. Mutant strains have been isolated which required tryptophan for growth (190). One strain was capable of synthesizing tryptophan only from indole, though it synthesized anthranilic acid, but was incapable of converting this to indole. Another strain was capable of synthesizing tryptophan from anthranilic acid via indole (192). A mutant, involving one mutant gene, required isoleucine and valine for growth (9). A strain was characterized by the loss of the ability to synthesize *p*-aminobenzoic acid; it differed from a normal strain by a single gene (189). In this manner, mutant strains were obtained requiring pyridoxine and thiamin (6), inositol (5), choline (71), and lysine (32). A mutant strain which differed from the normal by seven mutant genes required arginine (176); four of these genes were considered responsible for the synthesis of ornithine, two for the synthesis of citrulline from ornithine, and one for the final step from citrulline to arginine.

In a study of the effect of ultraviolet-induced mutation in *Aspergillus terreus* on the biochemical characteristics of the mutants, Lockwood *et al.* (102) reported the following findings. A total of 217 mutant strains were isolated which showed nine different types of biochemical and cultural responses. Among the 76 strains which showed no morphological changes, 59 showed no biochemical alteration, 13 produced more itaconic acid than the parent strain, and 4 produced no itaconic acid. Among the 141 strains, 42 were biochemically unaltered, 88 produced little acid, and 11 did not grow on the test medium. Fifteen strains produced considerable nonacidic unsaturated material. Seventeen strains appeared to produce no acid other than itaconic. It would seem that in a large number of strains the itaconic acid producing enzyme system was destroyed. In a few strains, the increase in ability to produce this acid indicates "preferential channelling of metabolism through this enzyme system."

According to Hollaender (68), *Penicillium notatum* spores irradiated at 2650 Å show, in general, a very wide distribution of variation in the yield of penicillin, with a predominance of low-yielding strains and some which produce practically no penicillin. However, occasionally a mutation was produced which gave an unusually high yield. Of 200 cultures tested, two were found of this type. The distribution of yield of cultures seemed to be definitely toward the lower side. Similarly, *Aspergillus terreus*, after irradiation showed thiamin deficiency. Ammonia or amino acid deficiency was observed when this mold was grown on a nitrate medium.

Observations show that in all mutations caused by irradiation the usual tendency is to produce changes which result in reduced metabolic activity.

According to Haberman and Ellsworth (56), the destruction of bacteria by x-ray treatment was found to occur semilogarithmically, indicating that

the rate of destruction was directly dependent on the numbers present at any given time. X-rays, acting in this manner, are comparable to disinfectants. In considering the effect of x-rays on colony morphology of bacteria, dissociation was assumed to take place when a quantum of x-ray energy was absorbed by an exposed cell, affecting a crucial point in the genetic mechanism.

Using cultures purified by single colony isolation, and administering care to reduce dissociation during the preparation of cell suspensions, Haberman and Ellsworth observed that, following the irradiation of non-proliferating cells of *Staphylococcus aureus* and *Serratia marcescens*, there occurred a greater tendency to form dissociants. More than 50% of all colonies of the latter organism were found to consist of different types of variants subsequent to irradiation for 25 minutes. Of these, two failed to ferment glycerol (using bromocresol purple as indicator during growth), three formed indole, and eight produced coagulation and peptonization of milk. Occasionally, streptococcus-like forms resulted from the treatment of *S. aureus* with x-rays. Loss of the ability to ferment glycerol might indicate that one or more enzymes involved in the oxidative phase of the fermentation process was damaged.

More definite information is reported by Gray and Tatum (51) regarding the change in the growth requirements of *Escherichia coli* and *Acetobacter melanogenum* as the result of x-ray treatment. While no variants of either organism were obtained following treatment with ultraviolet light, an x-ray treated culture of *E. coli* yielded two variant strains, one of which required biotin for growth, and the other, threonine. Four mutant strains were obtained from x-ray treated cultures of *A. melanogenum*, each requiring, respectively, serine or glycine, adenine or adenosine, glycine, and leucine. These mutations were attributed to special genes.

Roepke *et al.* (142) obtained, by single colony technique, with and without x-ray treatment, eight different mutant strains from a culture of *E. coli* which had been serially in a complete medium. These strains lost the ability to synthesize nicotinamide, thiamin, methionine, lysine, cystine, arginine, threonine or tryptophan. A ninth strain, isolated from x-ray treated cultures, gave growth in synthetic medium after the addition of either glycine or serine.

**Mutations Caused by Toxic Chemicals.**—Lips (100), discussing the similarities of mutations caused by x-ray treatment and certain chemical agents, makes reference to a finding by H. Stubbe, who, by treating the seeds of snapdragons (*Antirrhinum majus*) with chloralhydrate, copper chloride, 5% alcohol, pyridine, etc., succeeded in producing a definite dominant mutation. The plant, which, as the result of tissue degeneration, possessed puckered leaves.

Auerbach and Robson (1), experimenting with *Drosophila melanogaster*, tested several chemical substances (names not mentioned) for their ability



to produce gene mutations. Some of these produced mutations at a rate similar to that with x-rays. Searching for naturally occurring substances with the capacity to produce the same effect, these workers found that allyl isothiocyanate (mustard oil), which occurs naturally in a variety of plants (*Brassica nigra* and other *Cruciferae*), brought about the desired effect. The difference between the tested and control series was very marked. In addition, three sex-linked visible mutations were obtained in the two treated series, none in the controls. These observations with a naturally occurring chemical were considered of great interest for the light they throw on the nature of the gene and the process of mutation, and even greater importance for the theory of evolution.

**Mutations Caused by Sulfonamides.**—Emerson (40), discussing the unpublished results of Cushing, reported that by culturing *Neurospora* in the presence of sulfanilamide, a strain was obtained which grew in higher concentrations of the sulfanilamide than the normal strain. This characteristic was only partially maintained after a single subculture in the absence of the drug, and completely lost on outcrossing, indicating that this tolerance to sulfanilamide was not genetic. However, after growing this strain in still higher concentrations of sulfanilamide, a heterocaryon was obtained which contained some normal nuclei and some mutated nuclei. Isolated mutant strains were extremely tolerant to sulfanilamide.

These results indicate that the drug produces mutation of the cell nuclei in a manner comparable to the action of toxic agents on other cells. Unfortunately, data on biochemical changes associated with this mutation are not as yet available. It would be extremely interesting to learn whether this mutation affects the metabolism, particularly, of PAB, since it is stated that *Neurospora* synthesizes PAB and that a mutant strain requires it for growth, and since it has been claimed that sulfanilamide interferes with the metabolism of *p*-aminobenzoic acid. A biochemical study of bacterial mutation caused by sulfanilamide should provide an answer to this question.

According to Thomas and Chevais (194), intradermal injection of *o*-, *m*-, and *p*-aminobenzene sulfonamides into adult male fruit flies (*Drosophila*) affected the sperm cells and produced various mutations in the offspring produced from mating with virgin females. 4-Hydroxy-4'-aminodiphenylsulfone blocked mitosis in the blastomeres and did not produce mutations. The maximum number of mutations (1.75%) was obtained by mating the flies three to four days after the injection of sulfonamide. Mating during the first two days produced very few mutations (21).

**Mutations Caused by Artificially Colored Light.**—Ehrlich (37) makes reference to a finding by Engelman and Gaidukow who showed that *Oscillaria sancta* and *O. caldarium* developed pigmentation under the influence of artificially produced colored light. The pigmentation endured for months after removal to white light. Succeeding generations, which were not exposed to artificial color, also possessed it, which was thought to show the inheritability of the artificially produced pigmentation.

**Mutation Caused by Orthoquinoid Dyes.**—An observation which is related to the phenomenon of resistance is the loss of blepharoplast (kinetonucleus) by trypanosomes through the action of pyronine and similar orthoquinoid dyes.

Werbitzki (203), working in Ehrlich's laboratory, reported that, 40 hours after heavily infecting a mouse with trypanosomes, the injection of a pyronine solution (1 ml. of 1:150 dilution per 20 g. mouse) affected the trypanosomes to such an extent that from 40 to 60% lost their blepharoplast. In untreated infections, only about 5% contained no blepharoplast. Within 24 hours after the injection of 1 ml. of 1:1500 to 1:200 solution of orthoquinoid acridine dye, there were from 80 to 90% damaged cells. Following the progression of this effect he found that 4, 6, 8, and 12 hours after the injection of the dye, there were, respectively, from 10 to 12, 25 to 30, 40 to 50, and 70 to 80% of trypanosomes without blepharoplasts. The loss of this micronucleus, occupying a posterior position in the cell and away from the principal nucleus, was inheritable and associated with the development of resistance. It did not affect the motility, multiplication, or infectious property of trypanosomes. Atoxyl, antimony, acetoatoxyl, arsenophenylglycine, trypan red, trypan blue produced protoplasmic damages but had no effect on the blepharoplast. Similarly, dyes of parachinoid structure (containing the triphenyl-methyl group), such as trypan rose, parafuchsin, etc., damaged the protoplasm but had no effect on the blepharoplast. Kudicke (84) has established the fact that the increase in the number of the dye-resistant, blepharoplast-less trypanosomes is not due to the elimination of the susceptible blepharoplast-containing trypanosomes. Confirming Ehrlich's opinion, he states that the dye acts directly on the blepharoplast to eliminate it, producing a resistant strain. Resistance developed against arsenicals by another type of cellular change is explained in the same way.

Gonder (49), following up these observations, has presented evidence to show that the action of these dyes on *Trypanosoma lewisi* is to cause the blepharoplast to migrate from its position toward the principal macronucleus. If the dye acts slowly, the blepharoplast reaches its "origin" and takes refuge there before it is destroyed. In the majority of cases, however, it is destroyed during the migratory period. With *Trypanosoma brucei*, and perhaps with other trypanosomes, destruction by these dyes takes place extraordinarily fast. Gonder also reported that two other orthoquinoid dyes, triaminophenazonium chloride and triaminophenazelenonium chloride, are similarly effective.

Trypanosomes which had lost their blepharoplasts through the action of dyes were still without it after 400 mouse passages and were still resistant to them, which indicates the continued inheritance of degenerative mutation brought about by the agency of therapeutic agents.

**Comments.**—Toxic agents, whether physical or chemical, in the majority of cases produce genetic mutations. No doubt, in bacteria, fungi, and protozoa, certain variations do not lend themselves to visible observation so that a direct morphological link between genetic factors and cellular changes is difficult to establish. Biochemical alterations, therefore, must be determined in order to give a complete picture of both morphological and biochemical mutations as the result of the action of toxic or bacteriostatic agents.

Information is meager, except in the case of trypanosomes, on the correlation between genetic mutation and the resistance of mutants to the specific agent causing the mutations. For example, it would be helpful to know if the mutants produced by irradiation of *Neurospora crassa*, *Aspergillus terreus*, and *Penicillium notatum* are resistant to a second or third similar dosage of x-rays or ultraviolet light. Similarly, it would be of interest to know whether a second treatment with allyl isothiocyanide or with the isomers of aminobenzene sulfonamides exercises any or further effects on *Drosophila*. In other words, are these mutants resistant to these specific agents? Hypothetically, both x-ray and chemically induced mutants should resist subsequent action of these agents. However, a concrete answer must await further experimentation. The testing of mutants for resistance to other, structurally nonrelated toxic agents will not give the answer. Each agent is responsible for its own effect.

## 2. Observations on Phenomenon of Resistance

Two fundamental processes which a cell may undergo have been our present concern:

1. *A building-up mechanism which requires the incorporation and inheritance of genetic factors acquired by cross-fertilization, or, in an asexual cell, by the incorporation into the recipient recessive cell of cytoplasm material or of a catalyst of genetic nature from another strain of higher order but of the same species.*

2. *Degenerative mutations by which the cell undergoes hereditary losses as the consequence of the action of toxic agents.*

In the following discussion, the latter process will be our principal concern, since it is related to the development of resistance to toxic agents.

It may be assumed that toxic agents act on the vulnerable or susceptible sites of an organism. If the cell survives the experience, it may represent the constitution which is nonsusceptible to the specific agent. There are other explanations for the resistance of the surviving cell: (a) modification of the configuration of the susceptible cell proteins to a nonsusceptible

form, and/or (b) emergence of a new or an existing drug-insensitive metabolic pathway which may have occupied a secondary or minor role in the normal cell, and which acquires prominence as the drug-sensitive metabolism is suppressed. All these possibilities will be discussed in the following pages.

The development of tolerance was observed many centuries ago. But the process became a scientific subject with Ehrlich. The foundation of chemotherapy as a science, as well as the study of the phenomenon of resistance in microorganisms, have their beginnings in the studies of Ehrlich and his school. One of the most important discoveries by these investigators was that artificial development of resistance could be induced in trypanosomes and bacteria which they subjected to a thorough and comprehensive study. The principal aspects of these investigations will be outlined in another part of this section. At this point it may be mentioned that the development of resistance to arsenicals, for example, was attributed to a loss or diminution of affinity in the cells for the drugs. Gonder (49), a pupil of Ehrlich, showed that a resistant strain of trypanosome will not take on the vital staining by dyes of the orthoquinoid pattern, such as oxazine or selazin. In contrast, normal cells rapidly take on vital staining and eventually die. Similarly, Yorke *et al.* (216), Hawking (61), and Pedlow and Reiner (131) reported that normal trypanosomes removed arsenicals from a medium, whereas resistant trypanosomes failed to do so.

Since the discoveries of Ehrlich and his collaborators, numerous cases of the development of resistance to drugs and disinfectants have been reported. None of these supply definite information regarding the biochemical basis of the resistance reported. A partial review of these studies will be found in a paper by Severens and Tanner (164). The following are briefly discussed for particular reasons which will be evident from their treatment.

**Temporary Tolerance to Sodium Chloride.**—Doudoroff (34) reported the development of a reversible resistance to sodium chloride by *Escherichia coli*. By subjecting nondividing cells to gradually increasing concentrations of salt or to a single intermediate salt concentration, he obtained a salt-resistant strain. The number of bacteria capable of reproducing in a hitherto unfavorable salt environment increased with the period of exposure until a maximal value was reached. The rate and degree of individual acclimatization varied widely and were greater at higher than at lower temperatures. Maximal tolerance to saline solution was found during the early stationary phase of salt-free cultures, and minimal tolerance in the logarithmic and senescent phases. Acclimatized bacteria rapidly lost their acquired increased ability to reproduce upon return to a salt-free environment. Whatever may be the nature of this increased tolerance to salt, it is clear that it is of temporary, not of hereditary, nature.

**Permanent Resistance to Toxic Salts.**—Another study on the development of resistance to salts is reported by Severens and Tanner (164).

This study establishes the inheritance of the characters thus induced in bacteria. Using Chambers' micromanipulator, these workers made single cell cultures of *Salmonella pullorum*, *Eberthella typhosa*, and *Salmonella schotmuelleri*. Resistance was developed to sodium chloride, copper sulfate, and mercuric chloride by gradual, progressive cultivation in media containing increasing amounts of these agents. The resistance developed to each of these agents was specific, that is, there was no cross resistance. After 18 months of propagation in plain broth, involving 55 transfers, representing thousands of generations, all single cell cultures of resistant strains were unchanged with respect to resistance to these disinfectants. Thus, with the use of single cell technique, eliminating the possibility of selecting naturally resistant forms in the original culture, the development of permanent resistance in direct response to the action of chemicals has been demonstrated. As to what biochemical changes are associated with the resistance developed in these cases, there is no information.

**Inhibition of Capsule Formation.**—Hoogerheide (69) reported that adsorption of electrolytes on the surface of *Klebsiella pneumoniae* in general follows the lyotropic series and increases in the direction of  $\text{Li} < \text{Na} < \text{K}$ , etc. Such adsorption inhibits capsule formation, indicating that the enzymes responsible for the synthesis of capsular polysaccharides may be inhibited. It may be of interest to call attention to the inhibitory action that lithium exercises. According to Lindahl *et al.* (96), the Li ion inhibits the carbohydrate metabolism of sea urchin eggs, and Moore *et al.* (123a) reported that lithium reduces the rate of oxygen consumption of the eggs of sea urchin (*Strongylocentrotus purpuratus*) and sand dollar (*Dendraster excentricus*). The observations of Hoogerheide might be explained by supposing that lithium inhibits the carbohydrate metabolism of *K. pneumoniae* and thereby prevents the formation of capsular polysaccharide.

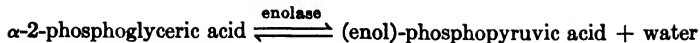
From the standpoint of the specific action of inhibitors on bacterial enzyme systems, the degradative changes that a pneumococcal cell undergoes is of interest. The virulent and encapsulated *Pneumococcus*, on repeated cultivation in peptone broth or acid medium, or, more specifically, in homologous immune serum, loses its virulence, as well as its ability to synthesize capsular polysaccharide. These changes signify that, under these conditions, the activities of the enzyme system responsible for the synthesis of type-specific carbohydrate is markedly suppressed and then eliminated as a result of the continuous inhibitory action of these conditions. Correlated with these facts is the marked difference in the respiratory activities of virulent and nonvirulent pneumococci observed by the writer (153). Stabilized nonvirulent *Pneumococcus* consumes markedly less

oxygen in the presence of glucose and lactate than do the virulent strains. The per cent of oxygen fixed as hydrogen peroxide was also markedly smaller in the avirulent variant than in the parent virulent strains. The most distinctive difference, however, was the failure of virulent *Pneumococcus*, in contrast to the strong activities of the avirulent variants, to oxidize ethyl and propyl alcohols. These results show that by a transformation from virulent to avirulent form, pneumococci undergo alterations in their respiratory enzyme activities.

**Inhibitory Action of Normal Sera on the Proteinase of *Streptococcus pyogenes*.**—The serological types of Lancefield's group A streptococci are characterized by a different type specific protein antigen, the "M substance." Elliott (39a) described a proteolytic enzyme elaborated by certain strains of streptococci which hydrolyzes the M substance, and, thereby, the serological type specificity of the strain is obliterated. He observed that human and guinea pig sera, in 10% concentration, did not inhibit the activity of the enzyme *in vitro*. In contrast, the activity was completely suppressed by rabbit and mouse sera. Mouse-virulent cultures of group A streptococci almost invariably were found to contain the M antigen, showing that the proteolytic enzyme is not formed under these conditions. Elliott also demonstrated that mouse passage of cultures, which originally were active producers of the enzyme, led to the suppression of the enzyme function and to a parallel increase in virulence and in production of M antigen. What is more important is the observation that the variant strain which loses its enzyme in this manner sustains this loss on subculture in artificial media.

On the basis of these observations, we are inclined to conclude that the loss of the proteolytic enzyme by the variant strain is not due simply to the inhibition by mouse serum of the extracellular enzyme, but to the actual combination of serum with the cell, which suppresses the activity of the enzyme system responsible for the production of the proteolytic agent. In other words, it is a suppression not only of the enzyme already produced, but more specifically, of the source that produces it. This consideration is of significance in understanding the mechanism of the development of resistance to drugs.

**Metabolic Differences between Fluoride-Susceptible and Fluoride-Resistant Bacteria.**—The development of resistance to sodium fluoride by microorganisms has been variously reported. It is known that fluoride is an inhibitor of enzyme systems. Of these the most thoroughly studied are the enzymes of fermentation. It is of particular interest that fluoride inhibits the following reaction:



For this reason all those reactions which lead to the formation of pyruvic acid, for example, glycolysis, are blocked by fluoride. Inhibition by fluoride is specific. Thus, a 0.05 *M* concentration has no effect on the mechanism by which dialyzed yeast extract transports phosphate to adenylic acid or adenosine from hexose diphosphate. In contrast, transphosphorylation from phosphoglyceric acid to adenylic acid is completely inhibited by 0.002 *M* sodium fluoride.

A report by Wiggert and Werkman (207) on the biochemical difference between fluoride-susceptible and fluoride-resistant strains of *Propionibacterium pentosaceum* is of immediate interest. These workers obtained two strains of this organism differing in susceptibility to sodium fluoride and in ability to ferment phosphoglyceric acid. The fluoride-resistant strain was incapable of fermenting phosphoglyceric acid, and, therefore, there appears a relationship between this and the accumulation of phosphoglyceric acid during the utilization of glucose by this strain. In contrast, the fluoride-susceptible strain was capable of fermenting phosphoglyceric acid. In addition, the latter strain was incapable, and the former was capable, of growing in fluoride. Fluoride-resistant strains were obtained by growing the organism in media containing fluoride, and also by isolating it from normal cultures grown under ordinary laboratory conditions. These results suggested to these investigators that this organism can dissimilate glucose by at least two paths; one of these is a probable by-passing of the phosphoglyceric acid metabolism by the resistant strain.

Nord and Mull (128b), discussing the mechanism of carbohydrate metabolism by fungi and bacteria, in contrast to the mechanism involving phosphorylation reactions, stated that carbohydrate dissimilation can occur in three ways: (a) by oxidation (without participation of phosphorus), (b) by splitting of carbon chains, and (c) by the detour of phosphorylation. Considering the ability of the fluoride-resistant organism to metabolize glucose and its inability to metabolize phosphoglyceric acid, it is important to point out that according to Nord and Mull, phosphoglyceric acid need not be considered as the indispensable mother substance of the abundantly isolated pyruvic acid and of the unchanged amounts of alcohol found. For example, in the course of fermentation by *Fusaria*, phosphoglyceric acid was neither isolated in the presence of fluoride nor utilized as a carbon source to any measurable extent. Phosphoglyceromutase, required for the transformation of 3-phosphoglyceric to 2-phosphoglyceric acid, seemed to be lacking in these organisms. This deficiency was considered responsible for the failure of enolase (if present) to act on 2-phosphoglyceric acid to form phosphopyruvic acid. [According to Pillai (133) the presence of adenosine triphosphate accelerates, in the absence of fluoride, the transformation from phosphoglyceric to phosphopyruvic acid. If the addition

of adenosine triphosphate enables the fluoride-resistant strain to utilize the phosphoglyceric acid, it might be possible to obtain a closer view of the resistance phenomenon.]

In the light of the observations of Nord and his collaborators, it would seem, however, that the fluoride-resistant strain may have experienced a "loss" of the enzyme, phosphoglyceromutase, or enolase, or both, and thereby was unable to metabolize phosphoglyceric acid. The ability of the same strain to metabolize glucose indicates that another metabolic pathway, other than the one involving the utilization of phosphoglyceric acid, was made use of. This pathway may be the one proposed by Nord and Mull. From the standpoint of the biology of the organisms, it may be that both processes are in operation in this type of organism to varying degrees. The suppression of one of the pathways leads to the increased use of the other during the development of resistance to fluoride.

**Relation of "Spontaneous" Mutations to Resistance.**—In a population of bacteria or microorganisms of various types, the existence of individual cells which are more resistant to inhibitors than others has been variously reported, *e. g.*, fluoride-resistant and fluoride-susceptible individuals, sulfonamide-resistant and sulfonamide-susceptible strains, blephoroplast-containing and blephoroplast-less trypanosomes, etc. The point to keep in mind is that these characteristics are produced in a given strain under natural growth conditions and also in an environment containing toxic agents. Either condition, in most cases, appears to produce the same characteristics.

There are also many instances where a population of bacteria contains individuals with varying synthesizing abilities. These have also been produced under natural conditions as well as under the influence of various toxic agents. It appears that in these cases the organism experiences a diminution of metabolic activity, which is inherited for many generations. Many examples have already been cited in which the effect can be traced to a toxic agent. As to the nature of natural degenerative mutations, or so-called "spontaneous" mutations, there is as yet no definite information. However, the fact that a natural product, such as allyl isothiocyanate, can produce a genetic mutation in *Drosophila*, and that dyes which are structurally related to alloxazine, etc., produce the same effect in trypanosomes as is produced occasionally under natural conditions, is of specific significance in explaining the nature of "spontaneous" mutations.

In our opinion it would seem that the natural metabolic environment gives rise to toxic products which, acting on specific enzyme systems, or on genes, produce mutants with resistance potentialities. Acting on such



cells, toxic agents may render them resistant during the course of a single contact. Kudicke, working in Ehrlich's laboratory, was able to produce atoxyl-resistant trypanosomes by one treatment with acridine dyes which, as will be discussed below, appears to act on flavoproteins. Intermediary or degradative products of riboflavin produced during growth might be among the many factors involved in the mechanism of "spontaneous" mutations.

### 3. Mechanism of Resistance to Sulfonamides

**Is *p*-Aminobenzoic Acid Related to Resistance?**—The resistance of certain strains of bacteria, particularly of *S. aureus*, to sulfonamides has been explained by assuming that during the development of resistance the organism acquires the ability to synthesize a greater quantity of *p*-aminobenzoic acid (PAB) (89, 90, 175).<sup>\*</sup> Before examining the validity of this assumption, it must be pointed out that the hypothetical enzyme system involved in the synthesis of PAB would first have to be freed from, or rendered resistant to, the inhibitory action of sulfonamides by some *special mechanism* so as to permit the subsequent synthesis and the accumulation of an antagonistic amount of PAB. Only acquisition of such a special mechanism capable of unhindered functioning could permit the increased synthesis of PAB, and therefore it would constitute the primary and basic mechanism of the phenomenon of resistance to sulfonamides and other antibacterial agents.

The claim that resistant strains of *S. aureus* synthesize greater amounts of PAB appears to be based on inadequate experimentation. The assumed aminobenzoic acid of these claims is an arylamine produced as an oxidation product of tryptophan; it is almost certainly structurally different from PAB. Bacterial (83) and animal (17, 73) systems are known to oxidize tryptophan to one or more arylamines, such as kynurenine (an aromatic amino acid in which the pyrrole ring of tryptophan has ruptured) and its various derivatives, or to *o*-aminobenzoic acid. In all of these oxidation products of tryptophan, the amino group is in the ortho position of the benzene nucleus. The formation of *p*-aminobenzoic acid from tryptophan or its derivatives would require the transposition of the amino group from

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<sup>\*</sup> According to Landy *et al.* (90), organisms may acquire resistance to sulfonamides without increasing their production of PAB, as shown by their studies on *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae*, and *Diplococcus pneumoniae*. Sulfonamide-resistant strains of these organisms failed to synthesize greater amounts of PAB than did their parent, nonresistant, strains. Some other resistance mechanism must be at work in the case of these organisms.

the ortho to the para position. As yet there is no evidence that bacterial or animal systems are capable of performing this feat.

In this connection a study by Tatum and Beadle (189) with *Neurospora*, which requires PAB for growth, is of particular interest. An attempt was made to trace the course of the synthesis of PAB by *Neurospora* and to determine the step that is blocked by the mutant gene. A number of substances were tested for their ability to replace PAB: *o*- and *m*-aminobenzoic acids, and compounds which did not have an aromatic nitrogen, were tested and found to be inactive. When the aromatic para amino group was replaced by Cl or OH, no activity could be detected. These findings show that a mutant strain of *Neurospora* could not convert these substances into PAB, lacking the enzyme system to do so. Normal strains were also incapable of performing this act. In the absence of added compounds, during a three-day growth period, a normal *Neurospora crassa* strain produced about 0.1  $\gamma$  PAB per 25 ml. of medium. In the presence of benzoic acid, *p*-hydroxybenzoic acid, or tyrosine, in concentrations up to 1 mg. per 25 ml., the amount of PAB produced was not detectably increased. These facts demonstrate that the normal strain, which is capable of synthesizing PAB for its growth needs, is incapable of introducing an amino group into the benzene ring. A similar observation with *Clostridium acetobutylicum*, strain S9, which is reported to require PAB for growth, is reported by Lampen and Peterson (87). They found that *o*-aminobenzoic acid did not replace PAB in growth experiments in a vitamin-free casein hydrolyzate medium containing biotin, tryptophan, and cystine, showing that this organism was likewise incapable of transposing the amino group from the ortho position to the para position.

**Tryptophan in Formation of Arylamines.**—Tryptophan is indispensable for the formation of arylamines in staphylococcal culture fluids. Tryptophan is an essential amino acid for the growth of *Staphylococcus aureus* (155). It must either be synthesized by this organism, or supplied to it. Staphylococci which are highly resistant to sulfonamides and grow in a *glucose-free* amino acid medium do not produce measurable amounts of arylamine. Susceptible staphylococci likewise do not produce arylamines under these conditions.

However, in the presence of tryptophan, in a concentration 25 to 100 times greater than the minimal amount ( $1 \times 10^{-4} M$ ) necessary for the growth of the exacting strains of staphylococci (155), both the resistant and susceptible strains produce large amounts of arylamine. In fact, strains susceptible to sulfonamides have produced larger amounts of arylamine than resistant strains. A calculation of the ratios of arylamine\* to growth show the following relationships: a value of 1.66 for resistant strains, and 2.2 and 2.6 for susceptible strains. That is, on the average, susceptible strains produced 44% more arylamine than the resistant ones.

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\* This ratio is based on the photoelectric colorimetric readings of a dye produced by diazotizing the centrifuged supernatant, clear culture fluid and coupling with a coupling agent, a method widely used in estimating sulfonamides in blood or urine. Growth was measured with the Klett-Summerson photoelectric colorimeter. Ratio = colorimetric reading/bacterial turbidity reading.

However, *in the presence of glucose and in the absence of added tryptophan*, a resistant strain was capable of producing a much larger amount of arylamine than a susceptible strain. This may indicate that, during the development of acquired or natural resistance to sulfonamides, the staphylococci develop the ability to metabolize glucose for the synthesis of larger amounts of tryptophan for growth; this synthesized tryptophan incidentally serves for the formation of arylamine. As discussed elsewhere, glucose-tryptophan metabolism antagonizes the inhibitory action of sulfonamides on *S. aureus*.

Indications are, then, that arylamine formation by staphylococci resistant to sulfonamides may be dependent on increased tryptophan metabolism. As will be discussed below, tryptophan metabolism, involving also a specialized glucose metabolism, appears to have a direct bearing on the mechanism of resistance to sulfonamides. The strains susceptible to sulfonamides lack the ability to utilize tryptophan, or cannot do so to a marked degree.

**Growth Independent of Arylamine.**—If the arylamine formed in staphylococcal culture media were PAB and if the latter were an essential metabolite, the amount of arylamine formed should have a direct relationship to growth, as would be expected from the Woods-Fildes theory. However, as indicated before, staphylococcal strains which are either resistant or susceptible to sulfonamides can grow without the formation of a measurable amount of arylamine. Furthermore, it has been observed that the amount of arylamine formed could be less under optimal and greater under unfavorable conditions of growth. In 10% casein hydrolyzate containing 0.5% glucose and other factors, maximum growth of a resistant *Staphylococcus* was obtained, with an arylamine to growth ratio of 0.96. On the other hand, in 0.07% casein hydrolyzate, growth was poor and the arylamine to growth ratio was 5.44 (155).

These observations have been corroborated by results from growth experiments in casein hydrolyzate containing different carbohydrates. The effect of glucose in aiding arylamine synthesis, as judged by the arylamine/growth ratio, was greater than for *d*-fructose, sucrose, *d*-mannitol, or trehalose. *d*-Galactose was without any effect on arylamine formation, despite the fact that growth was about 25% greater than in the medium containing glucose. In the absence of any carbohydrate, the arylamine/growth ratio was 0.39; in the presence of glucose, it was 1.33; and in the presence of *d*-galactose, it was 0.39. Growth in *d*-galactose was 180% greater than in carbohydrate-free medium; nevertheless, the amounts of arylamine formed were identical. Growth, furthermore, could take place

without the formation of arylamine. These data show no parallelism between the amount of arylamine formed and the growth of staphylococci.

**Absence of Correlation between Resistance and Arylamine.**—The question as to whether arylamine produced by resistant strains of staphylococci exercise any antagonism to sulfonamides must be considered. To do this we must assume that the measured arylamine is *p*-aminobenzoic acid and reevaluate the results as such. As shown before, growth of resistant *S. aureus* in a medium containing glucose was not inhibited by sulfonamides. But growth in the absence of glucose and in the presence of pyruvate was strongly inhibited by sulfonamides. Calculating the amount of arylamine formed under these conditions as PAB, the following relationships were obtained (155):

In the presence of glucose where growth was not inhibited, the molar ratio of sulfanilamide (SA) to calculated PAB was 2985, *i. e.*, the system contained 2985 molecules of calculated PAB to counteract one molecule of SA. In the absence of glucose and the presence of pyruvate where growth was inhibited 75%, we found a 21% smaller molar ratio of SA to calculated PAB, or a greater amount of calculated PAB than in the above system where there was no inhibition. These results show that the presence or absence of inhibition under these conditions is independent of the amount of arylamine calculated as PAB. Similarly, in the presence of glucose, the molar ratio of sulfathiazole (ST) to calculated PAB was 490. That is, the amount of calculated PAB was about 10 to 20 times smaller than required to counteract the amount of sulfathiazole present. Under these conditions, the growth should have been completely inhibited. Nevertheless, growth was not inhibited. In contrast, in the presence of pyruvate, the molar ratio of ST to calculated PAB was 22% smaller than in the former system. Nevertheless, the growth was inhibited by 70%.

A highly susceptible staphylococcal strain produced an amount of arylamine which, calculated as PAB, was several times greater than the amount required to counteract the inhibition of growth by sulfonamides; nevertheless, a high degree of inhibition prevailed.

The above results can only mean that the arylamine found in culture media is not PAB. In other words, the resistance to sulfonamides exercised by staphylococci cannot be accounted for by assuming an increased synthesis of PAB. The evidence is against such an assumption. The method used by us for the determination of arylamine in culture fluids was basically that of Bratton and Marshall (10) for the determination of sulfonamides. Using the Klett-Summerson photoelectric colorimeter, the method in our hands permitted the measurement of as little as 0.02 to 0.03  $\mu$ g. of arylamine per ml., calculated as PAB. Assuming that staphylococci produce PAB in amounts less than the above, we are forced to conclude that these amounts do not appear to play a significant part in the action of

sulfonamides on bacterial growth.\* On the basis of these observations, the following conclusions can be drawn:

1. The arylamine found in most bacterial culture fluids is an oxidation product of tryptophan, and is not PAB.
2. There is no parallelism between growth and the amount of arylamine formed.
3. Staphylococci resistant to sulfonamides can grow readily without producing arylamine.
4. In the presence of sufficient tryptophan, other susceptible strains of staphylococci are able to produce large amounts of arylamine without change in their behavior to sulfonamides.
5. Even if a fraction of arylamines formed were PAB, the amount is not of significance in influencing resistance or susceptibility of bacteria to sulfonamides.†

Thus, PAB *per se* is not an indispensable metabolite, and has nothing to do with the resistance or susceptibility of staphylococcal strains to sulfonamides.

Reference may also be made to an observation by Fox (47). He reported that a diazotizable substance is accumulated when *Escherichia coli* is grown in synthetic media containing bacteriostatic concentrations of sulfonamides. As Fox stated, this is not PAB; in fact, its production is prevented by amounts of PAB necessary to block the action of sulfonamides. The substance is not produced in the absence of sulfonamide nor when bacteriostasis does not occur. According to Stetten and Fox (181a), this substance is probably 2-hydroxy-5,6-diaminopyrazine. In our systems, the arylamine formed appears to be related to tryptophan. Our studies also demonstrated that sulfonamides interfere with the metabolism of tryptophan by *Staphylo-*

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\* According to Landy *et al.* (90), quantitative analysis of PAB in staphylococcal cultures by both the microbiological and colorimetric methods are of the same order of magnitude. Since the increase in diazotizable primary aromatic amine in cultures of resistant strains is related to the oxidation of tryptophan, and since this arylamine has been shown to have no bearing on the resistance mechanism, it appears that, if the interpretation of the results obtained by microbiological assays are valid, it should have been possible to corroborate them with the results obtained with classical chemical methods or at least with the chemical quantitative determination of total arylamine, and to compare inhibitions of growth by sulfonamides under various experimental conditions, as we have done. On these bases, the results obtained by the microbiological assay method, used by Landy *et al.* cannot be supported.

† Peterson and Peterson (132a), evaluating our results in the light of data presented by Landy *et al.* (89), state: "... the exclusion of PAB on the basis of unlike chemical properties and quantitative data does not appear to rest on too firm a basis because of the uncertainty regarding the properties and quantitative determination of bound PAB." Our examination of the data of Landy *et al.* shows that the statement by Peterson and Peterson is based on an inadequate analysis of the data. For, contrary to their statement, the data actually support our conclusions.

*coccus*, and that the interference was counteracted by tryptophan. Apparently, in Fox's experiments, similar relationships are in operation; the fact that the diazotizable substance is produced in the presence of bacteriostatic concentrations of sulfonamide indicates that the drug was interfering with the metabolism of certain substances resulting in the accumulation of diazotizable substance. Also, the fact that the diazotizable substance does not form in the presence of an amount of PAB necessary to block the action of sulfonamides indicates that this antagonist inhibits the enzymes which produce this substance in the presence of drug.

On the basis of our observations, it appears reasonable to conclude that sulfonamides partially deprive the cell of tryptophan by introducing an abnormal oxidation reaction which is either absent or negligible during the metabolism of bacteria in a growth medium free from sulfonamides. In the case of those strains which have acquired resistance to sulfonamides, this property of oxidizing tryptophan has greatly increased and becomes an acquired characteristic of the cell. The continued presence of sulfonamides is not, however, a required condition for increasing arylamine production. Numerous staphylococcal strains have been found which manifest great resistance or susceptibility to sulfonamides and are producers of large amounts of arylamine without having been exposed to resistance training in the presence of sulfonamides (155, 175). Evidently there are physiological factors other than sulfonamides which contribute to the oxidation of tryptophan to arylamine. Whether increased arylamine production by these strains is associated also with an increased ability to synthesize tryptophan remains to be demonstrated.

**Theoretical Considerations.**—In our opinion, the development of resistance to drugs may be, in certain cases, another manifestation of the process by which an inhibitory process brings about a variation in the parasitic cell. This variation, or phenomenon of resistance, by the use of the usual techniques has been claimed not to produce deep changes in the cell, *e. g.*, changes in antigenic structure (see part VII), virulence, colony form, morphology, biological activity, etc. Nevertheless, there may be a change in cell substances indicated by loss of affinity for certain drugs and loss of ability to stain with certain dyes; and certain definite changes in specific enzyme activity occur. We believe that either the cells undergo quantitative diminution in certain enzymes, or a profound configurational change takes place without augmentation of the quantity of the enzyme which is highly susceptible to drugs or dyes in the nonresistant state. This view is in harmony with the above-cited examples. It is, however, contrary to that proposed by Lodge and Hinshelwood (28, 103). They call

the resistance to drugs "bacterial immunity," the implication being that bacteria synthesize an ever-increasing amount of the enzyme upon which the drugs or dyes act; or, an increased synthesis of an antagonist takes place as the process of resistance progresses. They regard the development of resistance as a *process of adaptation* and imagine that when a certain function of the cell develops during the "adaptive" process, there occurs an expansion of certain protein patterns whereby an ordered structure expands by the accretion of new units. As an example, they cite the decomposition of arsine catalyzed by a surface of metallic arsenic, which increases in ratio to the arsenic liberated from arsine. This is, however, an exceptional case and does not set up a universal pattern for catalytic systems. For, the oxidation of ammonia by metallic oxides, the decomposition of hydrogen peroxide by colloidal platinum or catalase, or the decarboxylation of  $\alpha$ -keto acids or amino acids by enzymes does not contribute to the increase of catalysts. Indeed, the reaction products usually are inhibitory to the catalysts. A great number of similar examples could be cited to this effect.

The discrepancy between above ideas seems to lie in the fact that inhibitory actions leading to the development of resistance, as well as to bacterial variations, are compared to adaptive enzyme processes; the latter, as discussed in a previous section, have no valid experimental basis. Furthermore, since neither sulfonamides, nor inhibitory dyes, nor other similar drugs have been shown to function either as reactive substrates or as intermediary hydrogen carriers, comparison of the two processes as a basis for the elucidation of the mechanism of the development of resistance is apt to lead to faulty conclusions.

It is claimed that the development of resistance to sulfonamides by bacteria results in increased synthesis of PAB (89, 90, 175). If PAB is a metabolite for a specific enzyme or enzyme systems, the activity of which in normal bacteria is claimed to be interfered with by sulfonamides, its increased synthesis on continued exposure to the drug would be conceivable if the latter would stimulate and not interfere with the metabolism of PAB. This, however, contradicts the Woods-Fildes theory. If, on the other hand, we accept the Woods-Fildes theory that sulfonamides interfere with the metabolism of PAB, the continued blocking of the metabolism of PAB by the drugs, in accordance with the above-discussed mutative metabolic processes, would eventually markedly or practically completely eliminate the activity of the enzyme system involved in the metabolism of PAB. Should this happen it should then be expected that the organism would experience an ever-increasing susceptibility rather than resistance as the

result of the elimination of the production of the sulfonamide antagonist, PAB. On the other hand, to be consistent in our reasoning, it must be suggested that the complete obliteration of the metabolism of PAB would yield a strain highly resistant to the drug, for the hypothetically sulfonamide-susceptible enzyme would be expected to have been eliminated during the development of resistance. Should this be the course of events, PAB need, however, cease to be considered as an essential metabolite. For, following the development of resistance, the organism does not require PAB for growth. This of course would eliminate the assumption that PAB is an essential growth factor, and would therefore leave no room for the Woods-Fildes theory.

McIlwain (110) obtained pantoyltaurine-resistant strains of *Corynebacterium diphtheriae* without the use of the drug or other inhibitors by serial subcultures under the following conditions: (a) in the presence of much  $\beta$ -alanine and absence of pantothenate, and (b) in the presence of little  $\beta$ -alanine, but falling concentrations of pantothenate. Pantoyltaurine-sensitive strains were obtained by repeated subculture in the presence of high concentrations of pantothenate. Evidently, under these conditions, a selection of resistant and sensitive strains in particular media was effected. The selected typically resistant strain, for example, was capable of synthesizing pantothenate from  $\beta$ -alanine, and therefore did not require the former for growth. In contrast, the drug-sensitive strain was incapable of synthesizing pantothenate. This resistance of the former strains was attributed to an increased synthesis of pantothenate as an antagonist for pantoyltaurine. This conclusion of McIlwain does not appear to be valid. As shown earlier, when an organism is capable of synthesizing a growth factor, its synthesis from simpler intermediates is resistant to the action of the specific inhibitor. In contrast, the utilization of the preformed growth factor is highly sensitive to its inhibiting analogue. The resistant strain of McIlwain appears to be resistant to pantoyltaurine because the organism is able to synthesize pantothenate from  $\beta$ -alanine. Our interpretation is strengthened by the following observation of McIlwain that a pantoyltaurine-resistant strain of *Streptococcus pyogenes* did not synthesize pantothenate; in fact the pantothenate-exacting characteristics remained unaltered whether resistance was induced or natural. This resistance, therefore, involved metabolic pathways other than the pantoyltaurine-pantothenate relationship, which was shown to be susceptible to the inhibitory action of salicylate.

In connection with the above theoretical and experimental considerations, an observation by Woolley (209) is of interest. Woolley and White (210) had previously shown that pyrithiamin, the pyridine analogue of thiamin, selectively inhibited the growth of only those microorganisms which required thiamin for abundant growth. Nonsusceptible species did not owe their resistance to the production of thiamin or other antagonists of pyrithiamin.

After thirty transfers in increasing concentrations of pyrithiamin, Woolley obtained a pyrithiamin-resistant strain of *Endomyces vernalis* which grew in 25 times the amount of pyrithiamin which was sufficient to inhibit the growth of the parent strain half-maximally. In the absence of pyrithiamin, the resistant strain required 40 hours to produce the same amount of growth made by the parent strain in 24 hours.



The parent susceptible strain required only the pyrimidine portion of thiamin, pyrimidine alone was effective in counteracting the action of pyrithiamin (210). Woolley found that, as before, the pyrithiamin-resistant strain was still incapable of growing in the absence of thiamin or of the pyrimidine component of thiamin. In a skillful experiment, he observed that the resistant organism derived its pyrimidine by splitting pyrithiamin. The pyrimidine thus produced accounted for the 50% growth-promoting activity of pyrithiamin by the pyrithiamin-resistant strain. This organism protected itself, partially, by destroying pyrithiamin and making use of the liberated pyrimidine. Woolley stated that this reaction was not the sole means by which the resistant strain protected itself. These observations show that development of resistance to pyrithiamin does not produce an ability in the organism to synthesize thiamin or pyrimidine required for its growth and for counteracting pyrithiamin. [The presence of a large amount of pyrithiamin did not stimulate the synthesis of thiamin by *E. coli* (210).]

It would seem that, during the development of resistance, two processes, occurring concurrently in the metabolism of the parent strain, come to a climax when resistance to pyrithiamin is developed. The fact that in the absence of pyrithiamin the growth of the resistant strain progresses at a slower rate than that of the parent strain shows that the reactions responsible for this must have been continuously suppressed during the development of resistance. Simultaneously, the reaction responsible for the splitting of pyrithiamin (probably involving an oxidation-reduction reaction at the methylene linkage between pyrimidine and thiazole which may have been in operation at a very slow rate in the parent strain) has played an increased role during the development of resistance.

The above observations represent features comparable to the increased oxidation of tryptophan yielding greater amounts of an arylamine by *S. aureus* as the result of the development of resistance to sulfonamides (155). Recent results (unpublished data), obtained by the author with the collaboration of E. Steers, showed that the growth of a strain of *S. aureus*, resistant to sulfathiazole, in the absence of tryptophan, in comparison with the parent susceptible strain, was markedly slow. A time lapse of 40 to 120 hours was required to produce the same amount of growth as that of the parent strain. In the absence of tryptophan, the growth of the resistant strain was markedly inhibited by sulfathiazole. The gradual overcoming of this inhibition concurred with the period during which growth was accelerated and, therefore, the synthesis of tryptophan. In the presence of added tryptophan growth of the resistant strain was fast and was not inhibited by sulfathiazole; on the contrary, in the presence of sulfathiazole, growth was accelerated. It is, therefore, clear that, as discussed previously by Sevag and Green (155), tryptophan is an antagonist of sulfonamides, and its synthesis is suppressed by them. In these normal reactions, PAB has not been found to play any rôle.

The most plausible explanation of the process whereby resistance to drugs is developed lies in the degradative or modifying effects of inhibitors on vulnerable enzyme systems, with the concomitant emergence of other metabolic activities which may have occupied a secondary rôle in the non-resistant parasite.

**Alternate Metabolic Pathways.**—The facts regarding the development of resistance to sulfonamides by bacteria may, perhaps, be compared with those observed by Ehrlich and his school. Loss of affinity by re-

sistant bacteria to combine with drugs (indicated by the failure of drugs to exercise inhibitory action and by lack of stainability) may indicate: (a) certain configurational changes in the drug-susceptible specific enzyme proteins which may also involve changes in the activities of the enzyme to account for the resistance developed; or (b) the particular drug-susceptible enzyme has lost its role in the modified metabolism of the drug-resistant strain. In other words, the drug-resistant enzyme which did not play a significant role in the metabolism of the normal, susceptible strain may have gradually assumed a prominent role in the metabolism associated with the development of resistance. This may represent a compensatory process to meet metabolic requirements under adverse conditions. The answer to these possibilities must come from quantitative biochemical studies of the various changes which take place in the processes leading from a state of drug susceptibility to a state of drug resistance.

The development by bacteria of resistance to sulfonamides is observed in media containing glucose but not in its absence or in the presence of pyruvate (155). This indicates that the resistant strain has acquired a modified pathway to the metabolism of glucose, which is different from that used by the drug-susceptible parent strain. Studies on the rate of growth of staphylococcal strains show that the metabolism of glucose is an important factor, increasing growth several fold. In the absence of glucose, the growth rate is slower and of smaller magnitude. It is reasonable, therefore, to assume that inhibition of glucose metabolism by sulfonamides also results in inhibition of growth. Approaching the problem of the development of resistance to sulfonamides from this standpoint, we observed that, in the presence of glucose, sulfonamides fail to inhibit the growth of resistant *Staphylococcus aureus*. This was in contrast to the high degree of inhibition of growth of the susceptible *parent* strain. It was interesting also to observe that, while the growth of the resistant strain was not inhibited by sulfonamides in a medium containing glucose, it was strongly inhibited in the absence of glucose, or in the presence of pyruvate as a substitute for glucose. These results indicate that, during the development of resistance to drugs, the organism acquires the specialized ability of metabolizing glucose which, in some manner, by-passes the inhibitory action of the drugs. However, this is not a highly rigid specialization. For we have observed that after 100 or more daily subcultures on extract agar, free from added glucose or sulfonamides, the resistant mechanism shifted from glucose to amino acid metabolism. In other words, the organism now demonstrated resistance to the inhibitory action of sulfonamides in amino acid medium and susceptibility in the same medium containing glucose. Dur-

ing this interchange of the resistance process between glucose and amino acids, the high susceptibility of the resistant strain in a medium containing pyruvate did not undergo any change. In these processes, tryptophan metabolism appears to exercise a critical role. An analysis of the various factors involved in the resistance exercised by a highly susceptible staphylococcal strain showed the following facts: Tryptophan is a critical amino acid for the propagation of staphylococci; they must either synthesize it or be supplied with it. Sulfonamides interfere with the metabolism of tryptophan. Pantothenic acid, which in some manner mediates the synthesis of tryptophan from glucose and amino acids, singly, or, more effectively, in combination with riboflavin, enables tryptophan to counteract the inhibition of its synthesis or utilization by sulfonamides.\*

Related to this question is an observation by MacLeod (111, 112). Studying the methylene blue reducing activity of washed suspensions of a "sulfapyridine-fast" and its parent "susceptible" strain of *Pneumococcus*, he observed that the drug did not inhibit the reducing action of either strain in the presence of glucose. On the other hand, reduction of methylene blue by the susceptible strain was inhibited in the presence of glycerol, sodium lactate, and pyruvate. In contrast, the drug-fast strain was incapable of reducing methylene blue in the presence or absence of sulfapyridine, using any of the above 3-carbon substrates as hydrogen donors. These tests were carried out with nonproliferating, resting cells; therefore, it cannot with certainty be stated that the "drug-fast" strain would also demonstrate the corresponding property if the tests with the same substrates were carried out under growth conditions. However, it is important to observe that with the development of "drug-fastness," only those enzymes which were involved in the reduction of methylene blue in the presence of 3-carbon hydrogen donors were the ones susceptible to the action of drug, and only these enzymes lost the ability to reduce methylene blue subsequent to "drug fastness."

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\* Since in our experiments with *S. aureus* one of the final products of glucose metabolism is lactate, the observations of Hills (66) may be related to ours. In washed suspensions of *Proteus morgani*, grown with suboptimal supplies of pantothenate, the addition of pantothenate increased the aerobic metabolism of pyruvate to a greater extent than that of other substrates and the anaerobic metabolism of pyruvate more than that of nine other substrates tested. The substrates which gave increases in oxygen uptake approaching that with pyruvate were six C<sub>4</sub> and C<sub>5</sub> dicarboxylic acids, and lactate. In fermentation tests, no effect was observed on these, or of malate or glycerol, but all (lactate was not tested) were shown to increase the rate of fermentation of pyruvate. With four-carbon dicarboxylic acids, including malate but not succinate, the increase was several fold. With glucose as substrate, fermentation was practically unaffected by pantothenate and oxygen uptake was increased only in the later stages.

#### 4. Relation of Flavoproteins to Resistance

The development of resistance to drugs appears to be controlled by the ability of certain respiratory enzymes to resist the inhibitory action of drugs. Flavoprotein, which is involved in the metabolism of both glucose and certain of its breakdown products and of amino acids, appears to be one of these, as is indicated by the following observations: (a) Staphylococcal strains, highly susceptible to sulfonamides, are not inhibited by drugs when preformed riboflavin is included in the media (155). (b) Atabrine, which is structurally related to alloxazine, inhibits the activity of flavoprotein and is counteracted by riboflavin (55). (c) Acriflavine (proflavine), structurally related to alloxazine, inhibits the growth of *Escherichia coli* and of *Streptococcus pyogenes* in tryptic casein hydrolyzate, or it inactivates the hydrogen transport system, and is counteracted by riboflavin, phenazine, methylene blue, etc., which are structurally related to acriflavine (108). (d) Sulfanilamide inhibits the growth and respiration of *E. coli* but not in the presence of methylene blue (22). This indicates that sulfanilamide inhibits flavoprotein but that this inhibition is counteracted by methylene blue because of its greater affinity for flavoprotein. An inhibition of the cozymase dehydrogenase by sulfanilamide could not be expected to be counteracted by methylene blue in the absence of affinity between the dye and cozymase dehydrogenases. (e) Optochin inhibits the reduction of its structural relative, methylene blue, by *Pneumococcus* (148). (f) PAB counteracts the inhibition by atoxyl of the growth of *E. coli* (132).

What is more important is the observation by Madinaveitia (113) that the inhibitory actions of mepacrine, quinine, and methylene blue on the growth of *Lactobacillus casei* E are counteracted by riboflavin. In contrast, riboflavin had no effect on inhibitions by various nonrelated structures.




Thus, it may very well be that at least one of the sites of the resistance phenomenon is flavoprotein. The observations discussed above may be compared with those of Ehrlich and his collaborators on trypanosomes; a summary of these follows:

1. An arsenical-resistant strain of trypanosomes was developed which passed 350 times through normal mouse during a period of 2.5 years with its resistance unchanged (38, 39).

2. A fuchsin-resistant strain of trypanosomes was resistant against a series of triphenylmethane green, blue, violet, and red dyes, such as malachite green, methyl violet, night blue, etc., but was not resistant against arsenicals or orthoquinoid dyes such as pyronine, acridine, oxazine, selazin, and thiazine. Arsenical-resistant strains were resistant also against orthoquinoid dyes but not against triphenylmethane dyes (11, 37,

38, 124). A strain of trypanosomes could be made resistant to all three classes of compounds.

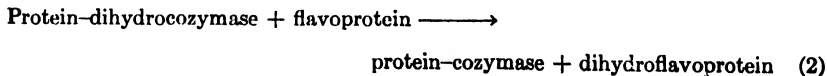
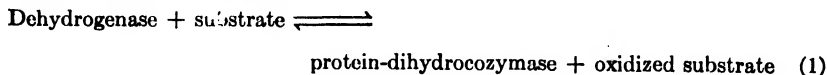
3. A strain of trypanosomes could be made resistant to arsenicals by one treatment with trypaflavin, an acridine dye. This occurred promptly, in contrast to repeated treatments with arsenicals (84).

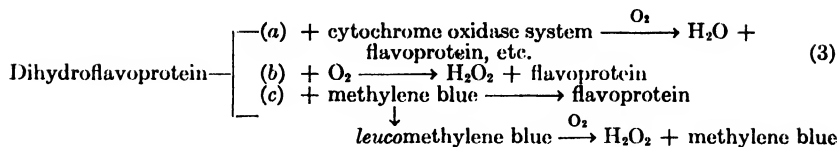
4. Atoxyl-resistant strains (or strains resistant to related arsenicals) were highly sensitive to arsenophenylglycine containing the  $\text{HOOC}-\text{CH}_2(\text{NH}_2)-$   group. That is, the sites susceptible to the former were different from those susceptible to the latter compound. (This is analogous to the high susceptibility to a sulfonamide containing the  $\text{H}_2\text{N}-\text{CH}_2-$   group by bacteria made resistant to sulfonamides containing the  $\text{H}_2\text{N}-$   group.)

5. Deep violet oxazine and selazine dyes stained normal trypanosomes but failed to do so with resistant trypanosomes. Normal cells underwent rapid vital staining with oxazine dye solution with subsequent morphological aberrations and destruction, causing the decolorization or reduction of the dye. In contrast, the dye had no staining effect on the resistant cells and was not decolorized, even when the cell died. While a 1:75,000 solution of selazine was effective against normal cells within 15 to 60 minutes, a resistant strain resisted a 1:300 to 1:1500 solution of the dye for 24 hours (49). These facts indicate that the affinities of trypanosomes for these dyes were abolished in some manner in the resistant strain. The fact that dead cell particles from normal cells were capable of, and those from resistant cells were incapable of, reducing the dyes may indicate the absence of the reducing system in the resistant cell substance.

6. Shiga (165), working with *Vibrio cholerae* in Ehrlich's laboratory, developed specific resistances to methylene blue (20-fold), new methylene blue (40-fold), trypanflavin (100-fold), and ethyl violet (100-fold). Testing for cross-resistance, he found that each agent had produced measurable mutual resistance in each strain. The strain which was 100-fold resistant against ethyl violet showed, however, least, or hardly any, resistance to the other three agents.

A structural similarity exists between orthoquinoid dyes (acridine, thiazine, oxazine, pyrrone, etc.), to which trypanosomes can be made resistant, and alloxazine, the principal component of the coenzyme of flavoproteins. Taking the dyes represented by the thiazine pattern, or, more specifically, methylene blue, as an example, the phenomenon of resistance may be approximated by the following scheme. Here, methylene blue is considered a hydrogen acceptor (or carrier) in the chain of reactions mediated by various respiratory enzymes:





According to the present concept, in order that the cozymase group (pyridine-adenine-dinucleotide) of the dehydrogenase should function as an enzyme, the reduced form must be oxidized. This appears to occur readily only by flavoproteins. The reduced flavoprotein must now in turn be oxidized. This is accomplished either directly by oxygen (in the absence of the cytochrome oxidase system) or through the cytochrome oxidase system, or by methylene blue in the case of isolated systems. Considering the last reaction indicated in the above scheme, the reduced methylene blue is oxidized, under aerobic conditions, by oxygen, accelerating the rate of oxygen uptake. Under anaerobic conditions, reduced methylene blue is the final product, as indicated by the disappearance of the blue color. In certain systems, xanthine oxidase, aldehyde oxidase, *d*-amino acid oxidase, *l*-amino acid oxidase, and flavoproteins themselves act as dehydrogenases, catalyzing, for example, the reduction of fumaric acid by one of several leuco dyes serving as hydrogen detonators, the products of the reaction being succinic acid and the oxidized or colored form of the dye; or catalyzing the oxidation of *l*-amino acids where oxygen or methylene blue acts as a hydrogen acceptor. A direct link between dehydrogenases (cozymase as coenzyme) and hydrogen acceptors (methylene blue, cytochrome oxidase system, or oxygen), requiring no flavoprotein, is not believed to exist.

The resistance of trypanosomes to thiazine dyes indicates that either the enzyme, flavoprotein, has undergone a configurational change with loss of affinity for the dye, or that the synthesis of this class of enzymes has been markedly suppressed by processes of bacterial variations, as previously discussed. The latter possibility could not, however, be supported too strongly if it were shown the present concept of the mechanism of oxidative reactions is rigid and does not offer alternative by-passes. Further critical and comprehensive quantitative studies are, therefore, necessary to determine whether (a) configurational changes of flavoproteins, or (b) reduction or marked diminution in the amount of these enzymes occurs in a resistant cell. Studies dealing with the effect of various degrees of riboflavin deficiencies in the bacterial cells *per se* on the respiratory and growth activities are very much needed. It is known that bacterial cells such as *Pneumococcus* can flourish without heme-containing enzymes such as cytochrome oxidase, catalase, and certain components of cytochrome.

Under controlled conditions Waring and Werkman (201) observed that, in the iron-deficient bacterial cell, certain iron-containing enzymes may be depleted or entirely absent without affecting the activity of other enzymes present. Iron deficiency apparently does not produce a general feebleness of the bacterial cell. At least no such feebleness was observed in the facultative organisms of the coli-aerogenes group. Studying the prolonged effect of 5% cyanide on yeast cells, Meissel (117) observed that the cells underwent plasmolysis, swelling, and shrinkage, and new variants were obtained. Apparently, by the suppression of cyanide-sensitive respiratory enzyme metabolism, different metabolic pathways were utilized with the emergence of new variants. Whether the suppression of flavoprotein activity can be accomplished with similar results remains to be investigated.

It has been reported (77) that when *p*-dimethylaminoazobenzene is fed to rats, causing hepatomas, the activity of flavin-containing enzymes in rat livers and tumors drops. The tumor-producing agent causes in rats a primary increase in urinary riboflavin excretion followed by a progressive decrease until markedly subnormal values are found. Whether microorganisms which develop resistance to sulfonamides and dyes undergo a similar impairment in riboflavin metabolism requires further investigation.

As stated above, optochin, structurally related to riboflavin or alloxazine, inhibits the ability of *Pneumococcus* to reduce methylene blue, and this organism has been shown to develop resistance to this drug. In view of the structural similarity, the inhibitory or resistance reactions can be considered as involving flavoproteins. This is significant in view of the fact that flavoprotein is one of the principal respiratory enzymes of pneumococci deficient in the cytochrome system, and that a great many pneumococcal strains are cyanide-insensitive. The oxygen consumed by the *Pneumococcus* is found to be fixed principally as hydrogen peroxide (152), which is presumably due to the oxidation of reduced flavoproteins by molecular oxygen. The action of sulfonamides on *Pneumococcus* appears to involve flavoproteins. This view is supported by the fact that in MacLeod's experiments type I *Pneumococcus* resistant to sulfapyridine lost the ability to reduce methylene blue in the presence of glycerol, lactate, or pyruvate as hydrogen donors, but not in the presence of glucose. This might indicate that there are several flavoproteins specific for the oxidation of each of the substrates mentioned, and that only those flavoproteins which are involved in the oxidation of C<sub>3</sub> hydrogen donors are involved in the resistance phenomenon, and not the one participating in the oxidation of glucose. Loss of the ability to reduce methylene blue by the resistant strain was associated with loss of the ability to form hydrogen peroxide. For it was

found that, during the growth of the parent, susceptible strain, large amounts of hydrogen peroxide were formed in contrast to negligible amounts formed by the resistant strain. As indicated in the above scheme of respiratory reactions, hydrogen peroxide forms when oxygen reacts with reduced flavoproteins.

In connection with these considerations is an observation by Davies and Hinshelwood (28). Working with *Aerobacter aerogenes*, the resistance developed to proflavine, comparable in structure pattern to riboflavin or alloxazine, rendered this organism equally resistant to methylene blue. This again may be interpreted as involving flavoprotein. Proflavine-trained cells were also found to acquire resistance to sulfonamide without losing it to proflavine. In contrast, the resistance developed to crystal violet, a triphenylmethane dye, did not confer resistance to proflavine, which indicates that crystal violet acts either on an enzyme other than flavoprotein, or that configurational changes wrought in flavoproteins to account for the resistance to crystal violet are not compatible with the structural pattern of proflavine and methylene blue.

Before concluding this discussion, it might be suggested that there is a relation between the processes and the nature of the enzyme underlying the resistance developed by trypanosomes to atoxyl and to dyes of similar structure and the counteraction by PAB of the inhibition of bacterial growth by atoxyl. This is in line with the fact that PAB is the essential component of anesthetics such as procain, and that the inhibitory action of sulfonamides has been likened to that of narcotics. Quastel (135) has shown that narcotics suppress the respiration of tissue. The origin of this effect has been traced to the flavoprotein link; thus the bridge between dehydrogenases and the cytochrome oxidase system is blocked, but, it is stated, neither dehydrogenases nor cytochrome are affected by narcotics.

In view of the above perspective, it might be that a certain "impairment" of flavoproteins takes place as resistance to drugs and dyes develops. It is also possible, however, that, as resistance develops, the living cell shunts or by-passes certain metabolic pathways and utilizes respiratory enzymes in other combinations without involving flavoproteins. This, however, is not in conformity with today's rigid concepts formulated on the basis of results obtained from isolated systems. Finally, it must be borne in mind that a search for the final answer to the phenomenon of resistance to drugs must not overlook the possible involvement of other respiratory enzymes, such as dehydrogenases, carboxylase, phosphatase, phosphorylase, cytochrome oxidase, and the cytochromes. Only a thoroughgoing comprehensive study can show the right way. Information obtained from such studies may, no



doubt, be of some value in explaining the increased tolerance developed in animal systems to numerous drugs, known to occur since early times.

### VII. Modification of Antigenic Specificity Accompanying the Development of Resistance

Degradative genetic mutations, *e. g.*, the degradation of type-specific capsular *Pneumococcus* into the nontype-specific, noncapsular variant, are accompanied by a modification of the antigenic specificity of a bacterial strain under the mutative influence of homologous immune serum or adverse environmental conditions. This degradation is accompanied by a loss of the ability to synthesize type-specific polysaccharide, desoxyribose nucleic acid, or a potent genetic substance present in it, and by the inability to cause infection in a host. This represents a type of degradation that does not appear to occur when a type-specific *Pneumococcus* develops resistance to a sulfonamide or to a dye. However, observations described earlier indicate that certain drug-susceptible groups are either markedly eliminated by a change in the configuration of the susceptible bacterial protein, or that this protein itself is no longer synthesized. Under these conditions it would be expected that the organism will undergo antigenic modification. There are as yet no experimental data on this point. The gathering of such data will require the determination of the specific modification accompanying the development of resistance and the use of immunological tests to determine the validity of this concept.

Long before immunological experiments with pneumococcus to bring about degradative variations were made, this type of experiment was carried out with trypanosomes in the laboratory of Ehrlich (37). The action of specific immune serum on a trypanosomal strain produced variants which serologically were different from the original strain. Ehrlich called this change *mutation*. Numerous experiments were cited by which it was shown that the development of resistance in trypanosomes to a specific dye or to arsenicals produced a strain which immunologically was found to be different from the parent strain, though both the parent and the resistant variant exercised the same pathogenicity. This appears to be comparable to the development of resistance in bacteria without loss of virulence and certain serological properties. However, the presence or absence of serological differences in these bacteria have not as yet been investigated from the standpoint of the drug-susceptible components of the organism.

Browning (11), working in Ehrlich's laboratory, demonstrated that when he immunized mice against a parafuchsin-resistant trypanosomal strain, the protection was specifically against this strain, and not against the normal strain or a strain made re-

sistant against atoxyl. Kudicke (84) found that trypanosomes, deprived of their blepharoplasts, or micronuclei, by the action of pyronine dye, were immunologically different from the strains from which the variants were obtained, though both strains exercised identical pathogenicity in the same species of susceptible host. Ehrlich reported that he was able to obtain variant strains under natural conditions which were identical in their infective properties, but which demonstrated from weak to strong resistance to trypan dyes. This immunological variation produced under natural conditions, or resulting from the development of resistance to drugs, or by the rapid action of specific immune serum, which differentiated the variant strain from the parent strain, without loss of pathogenicity, was called "serum-resistance" (*Serumfestigkeit*) by Ehrlich.

According to Ehrlich, Roehl, and Gulbransen (39), the above specific immune serum, like dyes and arsenicals, acts on those specific receptors which are directly responsible for the production of receptor-specific antibodies. These receptors exercise physiological activities of nutritional importance. Ehrlich called these *nutrizeptors*. The normal strain is well equipped with these nutritional receptors.

When a chemotherapeutic agent exercises inhibitory or injurious effect on trypanosomes these *nutrizeptors* exercising antigenic activity elicit specific antibodies which are capable of combining *in vitro* or *in vivo* with trypanosomes. These combinations produce a mutation resulting in the loss of the receptors and the formation of a new type of *nutrizeptors*, which are apparently nonsusceptible to the drugs and antigenically are different from the previous ones. This is considered evident from the fact that, following the immunization of the mouse with the mutant strain, reinfection with the original strain does not provide protection against the latter.

It follows from the above discussion that the trypanocidal action of the antibody does not depend on an immediate injury to the trypanosomes, but on disturbing the nutrition by combining with the corresponding receptors. If the trypanosomes under these conditions are able to procure new active groups (or a new metabolic pathway), they can survive this fate and prolong their existence. By changing the term *nutrizeptors* to *respiratory enzymes*, the whole concept of Ehrlich and his collaborators assumes modern significance. Our work with sulfonamides, penicillin, etc., however, has not assumed Ehrlichian proportions either in scope or in depth. Specific problems are yet to be investigated in order to obtain a comprehensive view of the action of antibacterial agents, an action which involves respiratory enzymes, as Ehrlich's findings imply and as we have demonstrated by numerous experimental observations.

### VIII. Conclusions

It has been the aim of the present study to present the action of toxic or bacteriostatic agents, mutations, resistance, and immunity as interrelated

processes in microorganisms. The experimental data pertaining to the action of toxic or bacteriostatic agents seem to show that their effects are primarily on the respiratory enzymes. These, as mediators of energy-producing systems and, thereby, of synthesizing processes, exercise an essential role in the biology of cells. Their inhibition precedes inhibition of multiplication of cells. These enzymes appear to be the site of the competitive reactions which characterize the antagonism between inhibitors and antagonists. A critical consideration of the experimental data shows that the action of inhibitors on endogenous synthesizing processes of cells is very much more readily overcome by a suitable antagonist than is the inhibition of the utilization of the preformed antagonists which function as growth factors. The studies on competitive reactions, though of great value in determining the specific nature of the substances whose utilization (or synthesis) is being hampered, do not, however, satisfactorily reveal the nature of the sites in the cell susceptible to the action of either inhibitors or antagonists. On theoretical and experimental grounds, we believe that the cell components upon which the sulfonamides act are the respiratory enzymes. From the standpoint of determining the nature of biochemical processes involved in the development of resistance to inhibitors, it is necessary that we obtain more specific information regarding the actual sites of interactions. In several instances of resistance to dyes and drugs, it seems that the cells have been deprived of the sites susceptible to these agents.

In view of the above considerations, we have been led to the analysis of the "adaptive" nature of enzymes, since the term "adaptation" has been frequently used to denote the phenomenon of resistance. Our analysis of the experimental data has failed to support this concept. If it were based on sound experimental results, it would mean that substrates are capable of stimulating the cells to synthesize new enzymes, or that the cells experience an increase in specific cell materials, which is tantamount to an evolutionary change from a lower to a higher form of living structure. In contrast, we believe that the substrates in these instances bring out a metabolic activity of enzymes already present in the cells, but hitherto not in use. The presence or absence of certain critical factors appear to condition the metabolism of "adaptive" enzyme substrates as well as of others structurally related.

The development of new strains of cells within a species, occupying a higher place in the scale of metabolic activities, is a biological event of note. This event appears to occur when a recessive cell acquires genetic factors from another cell of the same species of higher order of function and structure. In contrast, the biochemical changes which occur with the develop-

ment of resistance to toxic agents or unfavorable environmental conditions appear to be regressive processes. As a consequence of the suppressive effects of toxic agents, cells appear to undergo some sort of loss. Experimental data support this conclusion.

In transformations from a regressive type to a higher structural and functional type, increments in the immunological properties of the cells are evidenced. Adverse conditions have been shown to reverse these processes. Similarly, it is expected that differences in the immunological properties of the strains which are resistant or susceptible to drugs exist. No attempt has been made during recent times to determine the subtle nature of these differences. Ehrlich and his associates have, however, reported immunological differences between drug-resistant and drug-susceptible strains of trypanosomes. In studies with bacteria, it is essential that the nature of the specific factors involved in the resistance phenomena be established as a preliminary step to the determination of concurrent immunological differences.

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4-2

# BIOLOGICAL ANTAGONISMS BETWEEN STRUCTURALLY RELATED COMPOUNDS

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## I. Introduction

During the past few years a series of investigations has shown that compounds exist in nature or can be synthesized which are closely related in structure to various biologically active substances and which have the power of calling forth in various biological systems the signs of deficiency of the natural substance allied to them. It has seemed advisable at this time to bring together some of the facts which have been discovered in this new field in the hope, not only of summarizing existing knowledge, but also of providing a point of reference and departure for further studies.

All the papers bearing on this topic cannot be reviewed not only because of lack of space, but also because many interesting cases of this type of phenomenon have not been recognized as such by their discoverers. In several instances, data which seemed not to agree with modes of thinking current at the time of their publication have been found, on re-examination, to exhibit analogy with the present observations on competition be-

tween structurally related substances. Many of these older examples will have escaped the notice of the present author. One case of this sort will serve as illustration. Kuhn observed that the algal sex hormones were not two discrete substances, but rather, different mixtures of the same two (*cis*- and *trans*-dimethylcroctin) which functioned in both sexes simultaneously. The sex-determining factor was the ratio between these pigments (19). Since the compounds in question were structural analogues, it may be that one exerted a specific positive action which was antagonized by the other, and entirely negated if sufficient excess of the second were present. Other similar examples of reinterpretations of old data will be mentioned in the course of this review.

One of the first instances of the basic phenomenon with which we are concerned was found by Woods (49). Let us consider this case because it will bring into focus several points which will concern us throughout the discussion. Like most milestones in science this one, too, had its predecessors. The observations of Quastel and Wooldridge (36) on the competition between succinic acid and the related malonic acid were known to Woods and colored not only his thinking, but also much of that now current in the field. Clark's (3) views on the mechanism of drug action were based on competition between structurally similar substances and were already published. Findings such as those of Woolley *et al.* (63) on the competition between nicotinic acid and some of its analogues were made before they could be fitted into the present picture. However, the work of Woods, because of its relationship to practical therapy, was the one in which sufficient light was thrown on the subject to cast a shadow ahead.

Woods showed that the growth-inhibiting or bacteriostatic action of sulfanilamide against certain bacteria was reversed by small amounts of *p*-aminobenzoic acid, a structural analogue of the sulfonamide. The two compounds in question differed only in the nature of the acidic grouping since, in the former, this was a sulfonamide and in the latter a carboxyl. The antagonism between the two compounds was competitive, that is, the absolute amounts of the materials present were only of minor concern, and the deciding factor between antagonism and the lack of it was the ratio between the two substances. Evidence was adduced [which was later confirmed by direct isolation (38)] that *p*-aminobenzoic acid was an essential element in the metabolism of several microorganisms. The hypothesis was proposed, therefore, that the bacteriostatic effect of the sulfonamides was due to their competition with the metabolite *p*-aminobenzoic acid, with the consequent production of a deficiency of that metabolite.

The salient features with which we are concerned in this demonstration on the sulfonamides are as follows. First, the antagonism between the two substances was competitive; in other words, the biological effect of one of the pair of compounds was negated by the other partner—that is,

it was reversible. Second, one of the competitive pair of compounds was a metabolite—that is, it was a native, normally functioning constituent of the biological system. This metabolite might be synthesized by the organism or obtained from the external environment, in which case it was called a vitamin or growth factor. Third, a close structural similarity existed between the competing pair of compounds. We may say that sulfanilamide was an inhibitory structural analogue of *p*-aminobenzoic acid.

Following these observations on *p*-aminobenzoic acid, a number of vitamins and other metabolites have had their chemical structures altered in various ways, and appropriate biological tests have revealed that many of the analogues thus produced behave competitively with the related metabolite. In so doing, they brought about bacteriostasis, the appearance of specific deficiency signs in animals, the manifestation of certain new pharmacological effects, or the inhibition of definite enzymic reactions. Let us briefly survey existing facts concerning the inhibitory analogues which have been made in this fashion for a number of metabolites so that we shall be better able to consider other aspects of the problem.

## II. Compilation of Examples

**Thiamin.**—A variety of alterations of the thiamin molecule have led to the production of agents with competitive inhibitory action towards this vitamin. Pyriethiamin (64) which has the same structural formula as thiamin except that the sulfur atom of the vitamin has been replaced by  $-\text{CH}=\text{CH}-$  (*i. e.*, the thiazole ring has been exchanged for a pyridine grouping) was the first of these. It likewise was the first structural analogue to be used for the production of a deficiency disease in animals. Not only did it bring about these changes in animals, but it also produced inhibition of microbial growth (65). This action was reversible by thiamin, and selective in that it occurred only in the case of those species which required the vitamin or its component parts as a growth factor (65). Oxythiamin, *i. e.*, 2-methyl-4-hydroxy-5-pyrimidylmethyl-4-methyl-5-hydroxy-ethylthiazolium chloride (44) has been found to compete with thiamin in animals and microbial species. A benzenoid analogue of the vitamin, *o*-aminobenzylmethylthiazolium chloride (39) in which the pyrimidine ring rather than the thiazole (as in pyriethiamin) has been modified, inhibited the enzymic splitting of thiamin by a factor found in certain fish (50).

**Riboflavin.**—Several different analogues have been found to cause reversible inhibition of microbial growth or the production of signs of riboflavin deficiency in animals. These include 2,4-diamino-7,8-dimethyl-10-

ribityl-5,10-dihydrophenazine (53), 6,7-dichloro-9-ribitylisoalloxazine (20), and 5,6-dimethyl-9-ribityl-isoalloxazine (7).

**Nicotinic Acid.**—Pyridine-3-sulfonic acid (24) has caused reversible inhibition of growth of a few bacterial species, but has been found innocuous in mice (66). On the other hand, 3-acetylpyridine brought about reversible signs of nicotinic acid deficiency in mice (56) and dogs (63), but not in microbial species (56). One cannot but wonder whether the pelagragenic agent in corn (17) may not be such an analogue of nicotinic acid with a similar specificity for animals.

**Pantothenic Acid.**—A number of different types of inhibitory structural analogues of pantothenic acid have been synthesized. The first was thiopanic acid or pantoyltaurine ( $\alpha,\gamma$ -dihydroxy- $\beta,\beta$ -dimethylbutyryltaurine) (21, 26, 41), which was related to the vitamin somewhat as sulfanilamide was related to *p*-aminobenzoic acid. Various modifications of this basic molecule resulted in compounds of varying activity (1) much as was the case in the sulfonamide series. Exchange of the  $-\text{CH}_2\text{CH}_2\text{COOH}$  portion of the molecule for alkyl or hydroxyalkyl groups (43) produced a second class of pantothenic acid antagonists. Alteration in the number of carbon atoms (26, 35), or in the branching of the main carbon chain of the vitamin, likewise led to a few compounds of low inhibitory potency. Finally, replacement of the carboxyl group with a phenyl ketone grouping led to a pantothenic acid inhibitor active against all microbial species tested (59). With the exception of *p*-aminobenzoic acid, more antagonists to pantothenic acid have been studied than for any other metabolite.

**Biotin.**—Representatives of two classes of inhibitory analogues of biotin have been synthesized and shown to function in bacteriostasis. In one (8), the sulfur atom was replaced by  $\text{CH}=\text{CH}-$ , and the length of the side chain was varied. In the other, the sulfur atom was removed leaving two side chains rather than a thiophane ring (5, 23). It was of interest that this latter type of compound was bacteriostatic for some organisms, but a growth stimulant for others. In the latter case, the compound was converted into biotin by the microorganism through addition of an S atom (4).

**Ascorbic Acid.**—Glucoascorbic acid, 2,3-enediol-*d*-glucoheptono-1,4-lactone, has been shown to call forth signs similar to those seen in scurvy (61).

***p*-Aminobenzoic Acid.**—Because of the widespread clinical use of the sulfonamides, and the belief that the action of these drugs is connected with *p*-aminobenzoic acid, a large number of structural analogues of the latter metabolite have been examined. The therapeutically active derivatives of *p*-aminobenzoic acid all may be viewed as substances in which the carboxyl group of the metabolite has been replaced by a sulfonamide or

one of a number of substituted sulfonamide radicals. In the case of arsenical drugs such as carbarsone (*p*-carbamidobenzenearsonic acid), the substitution has been an acidic group containing arsenic instead of sulfur. Frequently it has been desirable to modify the  $\text{NH}_2$  group in addition to the carboxyl in order to achieve certain properties. Examples of this may be seen in sulfasuxidine (succinylsulfathiazole) or in carbarsone. Study of variations in the composition of the sulfonamide part of the molecule has led to the hypothesis that bacteriostatic activity is correlated with the degree of ionization of the molecule (2). Since there are several excellent reviews which deal extensively with the sulfonamide question (see, for example, 13), a discussion of the subject would be redundant here.

The structure of *p*-aminobenzoic acid has been modified in other ways than those just referred to in the preceding paragraph, with the attendant production of bacteriostatic agents. Certain substitutions in the carboxyl group (other than those mentioned) such as the exchange of  $-\text{CONH}_2$  for  $\text{COOH}$  (15), or alterations in the atoms attached to the benzene ring, such as the replacement of  $-\text{H}$  by halogen (67), have been found effective. Likewise, exchange of one or more carbon atoms of the benzene nucleus for atoms such as nitrogen or sulfur have resulted in the production of competitors to *p*-aminobenzoic acid. A comprehensive study of the relative effectiveness of various types of structural alteration of *p*-aminobenzoic acid was made by Johnson *et al.* (16).

**Amino Acids.**—A general type of structural change which would convert  $\alpha$ -amino acids into bacteriostatic compounds was the substitution of a sulfonic acid radical for the carboxyl group (25). In the case of individual amino acids, various structural modifications such as the replacement of the sulfur of methionine by oxygen (37), or of the  $\beta$ -H of  $\beta$ -alanine by  $\text{CH}_3$  (to yield  $\beta$ -aminobutyric acid) (32), or the conversion of tryptophan to indoleacrylic acid (10) have been found to yield growth-inhibitory agents.

Of interest in this respect are the antagonisms which have been observed between pairs of naturally occurring amino acids. In many cases the members of an antagonistic pair may be viewed as structural analogues. For example, the competition between glycine and alanine (42) and between arginine and lysine (6) may be cited. The latter example was one in which a mutant strain of *Neurospora*, which required lysine for growth, was competitively inhibited in development by arginine. The parent strain of *Neurospora*, which did not require an external supply of lysine, was not inhibited in growth by arginine. This situation, however, should not be surprising when it is realized (see page 137) that structurally



analogous compounds frequently are harmful only to organisms which require the related metabolite as a nutritive essential. If this manner of viewing the antagonisms exhibited by certain amino acids is valid, it calls attention to the fact that the phenomenon of antagonism between structurally related compounds may be of significance in natural processes.

**Porphyryns.**—Certain of the naturally occurring porphyrins have been shown to behave competitively with iron protoporphyrin (11). Here, as in the paragraph above, the coexistence of the two competitive structural analogues in the same organisms was considered to have some implication in the mode of action of the materials biologically.

**Purines.**—Benzimidazole, which may be derived from purine by exchanging the pyrimidine portion of the latter for a benzene ring, has been found to compete with adenine and guanine in bacterial growth (52). Similarly, the triazolopyrimidines, in which the triazine ring has been substituted for the imidazole ring of the purines, competed with these metabolites in the growth of certain bacteria (37).

**Vitamin K.**—There are several types of inhibitory structural analogues of vitamin K. In some instances, however, the type of relationship which exists between the vitamin and the analogue is not so well defined as in the case of, let us say, thiamin and pyrithiamin. As a result, some caution must be exercised in integration of the data with those obtained in other competitive pairs. 3, 3'-Methylenebis-(4-hydroxycoumarin) and a number of its derivatives have been observed to cause signs associated with vitamin K deficiency in several species of animals (33). These signs were reversible by vitamin K (*i. e.*, 2-methylnaphthoquinone). The structural alteration in passing from vitamin K to 3,3'-methylenebis-(4-hydroxycoumarin) involved (among other things) the substitution of an oxygen atom for a carbon atom in the ring system. It has been shown that the substitution of sulfur or nitrogen rather than oxygen for this carbon atom resulted in compounds which produced some of the signs associated with deficiency of vitamin K (31). Certain alterations of the ring substituents in the coumarin could also be made without abolition of the pharmacological effects of the agent (34).

The antibiotic pigment, iodinin, which has more or less close structural resemblance to vitamin K, has been shown to be antagonized by this vitamin in so far as its effects on bacterial growth were concerned (27). Iodinin was believed to be a dihydroxyphenazine oxide, and could be viewed as a relative of vitamin K in which carbon atoms 1 and 4 had been replaced by nitrogen atoms, and the side chain converted into a benzenoid

ring. It would be of interest to learn whether this compound would cause signs of vitamin K deficiency in animals.

$\alpha$ -Tocopherol quinone, which has many points of structural similarity to vitamin K<sub>1</sub>, has been reported to cause hemorrhages localized in the reproductive tract of the pregnant mouse (57). Although this sign was not associated with the characteristic lengthening of prothrombin time of the plasma seen in vitamin K deficiency, it was preventable by very small doses of vitamin K. The mere reversal of the hemorrhage by the vitamin may not be conclusive since toxic doses of vitamin A have been combated with vitamin K (22); but the fact that this reversible hemorrhage resulted from the administration of a close structural analogue of vitamin K, makes it justifiable to include  $\alpha$ -tocopherol quinone among those agents classed as inhibitory analogues of this vitamin.

A potent fungistatic agent, 2,3-dichloronaphthoquinone (47), has recently been recognized as the structural analogue of vitamin K, and its inhibitory action on the growth of yeasts has been found reversible, competitively, by small quantities of that vitamin (58). As in the case of the coumarin derivatives discussed above, more of the vitamin K than of the dichloroquinone was required to cause reversal. It will be pointed out later that this is contrary to the usual situation in which the amount of metabolite needed to nullify the effect of an analogue is much less than the quantity of analogue present.

**Tocopherols.**—Search for a structural analogue of vitamin E, which would cause in animals signs of deficiency of this vitamin, led to the discovery that  $\alpha$ -tocopherol quinone brought about selectively in pregnant mice some of the signs of tocopherol deficiency (57). No reversal was possible by tocopherol, but vitamin K was found to be an effective antagonistic agent (see vitamin K above). The quinone was related structurally both to tocopherol and to vitamin K.

**Miscellaneous Metabolites.**—By careful perusal of the literature both current and past, it is possible to find several additional examples of the fact that compounds related structurally in certain ways to various metabolites are able to cause evidences of deficiency of these same metabolites in appropriate biological systems. Since this review does not purport to be exhaustive, only a few additional examples will be mentioned. In view of the fact that the androgens and estrogens occurring naturally are structural analogues, one of the other, and that in several respects the effect of the one may be counteracted by the admission of the other, it has been suggested (14, 60) that the action of these sex hormones is bound up with their structural similarity and competitive properties. The

normal occurrence of androgen and estrogen in the same individual has contributed to this view. The case of *cis*- and *trans*-dimethylcrocin in the role of sex hormones for algae was indicated at the outset of this review (19). Instances of competitive behavior between the plant hormone indoleacetic acid and the related phenylbutyric acid have been described (40). Cases of the inhibition of enzyme action by compounds related in structure to the normal substrate, such as the classical retardation of succinic dehydrogenase by malonate, are well known.

### III. Generalizations on Types of Structural Change Which Will Convert Metabolites into Inhibitory Analogues

Although it might seem, from the large number of compounds which have been found to behave competitively with various metabolites, that any alteration in the structure of a biologically active compound is sufficient to convert it into an antagonistic agent, this is actually not so. It has been pointed out that many structural analogues of various vitamins have been tested and found to be without inhibitory action (55). Nevertheless, there seems to be no single, specific way in which the structure must be modified in order to realize such an agent. For example, as will be evident from the foregoing citations, several distinct types of structural change applied to the same metabolite may give rise to antagonistic agents. It is of interest that for a given metabolite, one type of analogue may have biological effects rather different from those of a second class of structural relative. For example, 3,3'-methylenebis-(4-hydroxycoumarin) and  $\alpha$ -tocopherol quinone differ in their action on animals although they are both analogues of vitamin K. The difference in pharmacological effect is quite pronounced but despite this, the manifestations of each analogue are similar to those seen in deficiency of the related metabolite. Apparently all the signs of deficiency are not called forth by both types of analogue, and some elicited by one type are not produced by the second.

Generalizations have been made (55, 60) about the kind of alteration of the molecule which will result in the production of antagonistic analogues. It has been emphasized that these are only the first attempts in this direction and that the final picture may differ considerably from this preliminary one based on limited data. The inductions have, however, demonstrated their value in some small capacity by fostering the prediction of several new analogues which acted against various metabolites.

According to these generalizations the first method of converting a metabolite into an inhibitory agent was the exchange of some other group

for a carboxyl. This new substituent could be sulfonamide as in the case of the sulfanilamide drugs, or a ketonic group as in the case of acetylpyridine or phenylpantothenone.

The second general method involved the exchange of one or more atoms in the ring of a cyclic metabolite for different atoms. There are many examples of this class of inhibitors, but pyriethiamin and the phenazine analogue of riboflavin will serve as illustrations.

There were in addition several miscellaneous ways of altering structures in order to produce antagonists. Inhibitory structural analogues such as glucoascorbic acid and desthiobiotin serve to illustrate some of these.

#### IV. Some General Aspects of Inhibition by Structurally Related Compounds

A number of features of the general problem of biological antagonism produced by structurally related compounds should be considered. Few, if any, of the inductions which will be discussed under this topic will be found free of exceptions. It is doubtful whether any one characteristic can be set up as the criterion to determine what shall be considered a part of this biological phenomenon, and what shall be excluded. The situation is similar to that involved in discussions of the features of enzyme action for there too exceptions to most criteria can be found, and one must content himself with the description of aspects of the phenomenon which seem to be common to a majority of the individual examples.

In many cases it has been found that the ability of a structural analogue to inhibit a biological system (usually a growing bacterial culture) is related to the need of the organism for an external supply of the metabolite to which the agent is related. For example, thiopanic acid caused inhibition of growth of several bacterial species which required pantothenic acid as a growth factor, but was not toxic to several species which grew well without pantothenic acid in the medium (41). Presumably, the latter group met their needs for this vitamin by synthesis inside the individual cells. The dependence of inhibition of growth on need for the vitamin was seen even more clearly in the case of pyriethiamin (65). While this relationship has been observed with several types of antagonists, there are some notable exceptions. A case in point is the sulfonamide drugs in which inhibition of bacterial growth is observed whether or not the organism in question needs *p*-aminobenzoic acid in the medium. A similar situation occurs with benzimidazole (52), for this agent inhibits microbial growth irrespective of nutritional requirements of the organisms for purines.

Midway between these extremes of dependence of effect on nutritional requirement are examples of antagonists which affect all species, but for which the action is reversed by the metabolite only in the forms requiring the metabolite as a nutrient. Illustrations of this may be seen with glucoascorbic acid (51, 61) and with phenylpantothenone (59). Both of these compounds produced signs associated with deficiency (of ascorbic acid or of pantothenic acid, respectively) in all species tested. This action was prevented by the related vitamins only in those forms which required them as nutritional essentials. It seems probable that an understanding of the reasons why these situations exist would add to our knowledge of the utilization of the vitamins in metabolism.

Generally the amount of inhibitor required to produce an effect is much greater than the quantity of metabolite needed to reverse that effect. Inhibition indices (65), or antibacterial indices (26), which are the quotient of concentration of inhibitor divided by concentration of metabolite necessary to neutralize the effect, have been calculated for most competitive pairs, and have been found quite large, usually from 100 to 10,000 or even greater. Failure to recognize the large magnitude of the inhibition index has hampered some of the work with antagonistic analogues, especially with animals (see, for example, 46).

Exceptions to this generalization are rare, but three are known: 3,3'-methylenebis-(4-hydroxycoumarin) (33), 2,3-dichloronaphthoquinone (58), and benzimidazole (52). In these instances the index was less than one. It may be noteworthy that in these cases the amount of analogue required to produce an effect was very large in comparison to the quantity of the related metabolite present in the organisms. For example, about 2 mg. of the coumarin was required to affect a rat, and about 25 mg. of vitamin K to overcome this dose, whereas a rat probably contains much less than 1 mg. of the vitamin. If the inhibition index is approximately 0.1, one would expect a rat containing, let us say, 0.5 mg. of vitamin K to be influenced by 0.05 mg. of the coumarin. Here, too, an understanding of the underlying mechanism might have theoretical value.

It has been found that the inhibition index for the same inhibitor-metabolite pair is not identical for various species, and that relatively large differences in magnitude occur.

In the case of several inhibitory analogues it is possible to achieve reversal of their effects not only with the related metabolite but also with biologically occurring compounds with no structural relationship to the inhibitor or the metabolite. Thus, the bacteriostatic effects of the sulfonamides may be reversed not only by *p*-aminobenzoic acid, but also by

methionine (12) or adenine (30). Also, the action of phenylpantothenone may be counteracted by pantothenic acid, glutamic acid, or glycine (58a); and that of glucoascorbic acid may be nullified by ascorbic acid or by a substance found in green plants which has few of the properties of that vitamin (61). If the pellagragenic substance in corn (17) should prove to be an analogue of nicotinic acid, it would be of interest from this angle, since the toxic effects of corn are overcome either by small amounts of nicotinic acid or by large amounts of tryptophan (18). It is not certain whether this antagonism by structurally unrelated compounds is general for all metabolite analogues. Usually the amount of these reversing substances which are not related structurally to the inhibitory agent is quite large in comparison to an effective dose of the analogous metabolite.

As pointed out in the beginning of this review, the relationship between metabolite and structurally related antagonist is usually competitive, that is, the effect of the antagonistic agent is dependent not on the absolute amount present, but rather on the ratio between the amounts of inhibitor and of metabolite. Nevertheless there are cases known in which this is not strictly true, and yet it seems only logical to include these instances under the phenomenon of competitive antagonism between analogous compounds. An example is found with 3,3'-methylenebis-(4-hydroxycoumarin). The inhibition ratio is not constant for varying levels of the drug, and furthermore, as the concentration of the coumarin is increased, a point is soon reached beyond which vitamin K does not reverse the action. The amounts of vitamin and of antagonist, however, are so large that secondary deleterious effects of both of the compounds may obscure the results. Nevertheless, the coumarin is related structurally to vitamin K and does call forth characteristic signs of vitamin K deficiency, which are preventable by the vitamin.

There is the feeling in some quarters that the ability of the related vitamin to reverse the manifestations of a structural analogue must be taken as the criterion of the phenomenon we are discussing. This test is not necessarily foolproof. In the absence of such reversal it is indeed difficult to say that the agent in question acts by the production of a deficiency of the metabolite. However, it must be remembered that *p*-aminobenzoic acid will not reverse the bacteriostasis produced by the sulfonamides in some bacteria (45). Furthermore, while the growth-inhibiting properties of phenylpantothenone may be nullified by pantothenic acid in some microorganisms, they cannot in others (59); and glucoascorbic acid may be rendered noninjurious by vitamin C in guinea pigs, but not in mice (51, 61).

Finally, a given analogue of any one metabolite may be inhibitory in one kind of organism, and innocuous in another, even though the metabolite is known to function in both kinds. For example, the sulfonic acid derivatives of the vitamins do not produce signs of deficiency in most animal species, while they are quite effective in this respect with micro-organisms (66). From the information now at hand it would seem that the reasons for such relations are varied. In some cases the agent is rapidly excreted or actually destroyed, but for the most part the mechanism is unknown.

## V. <sup>orig.</sup>Antagonism between Structurally Related Drugs

The antagonist relationship between structurally similar compounds is not limited to cases in which one of the pair of participants is a metabolite. Both partners may be foreign to the biological system. For example, the pharmacological manifestations of morphine may be nullified by allylnormorphine (48). Several additional instances are known in the field of pharmacology, but since some of these may be found catalogued by Clark (3) they need not concern us further here.

## VI. Implications for Pharmacology

Since the intensive investigation of competitive inhibitory effects of structural analogues began with the observations on *p*-aminobenzoic acid and the sulfonamides, it is perhaps only natural that subsequent work has had a strong flavor of bacteriostasis. Moreover, the fact that the growing microbial culture represents one of the most accessible biological systems for investigation of the phenomenon of competition, has tended to concentrate attention on the possible use of metabolite analogues in the chemotherapy of infectious diseases. The possibility of producing new types of agents for such a purpose has been demonstrated by McIlwain and Hawking (29), who showed that sufficiently large doses of thiopanic acid (pantoyltaurine) could protect rats against lethal numbers of hemolytic streptococci. The compound was too readily excreted to make it a desirable therapeutic agent, but it is not beyond hope that some antagonistic metabolite analogue may prove of value in this regard (55).

There is reason to believe that the phenomenon of competitive inhibition between structurally related compounds may find broader application than merely to that section of pharmacology which deals with chemotherapy of infectious diseases. Woolley (55) has shown that some of the pharmacological effects of new compounds can be predicted from knowl-

edge of the signs of deficiency of various vitamins. It was pointed out that a variety of more or less specific signs were known to result from vitamin or hormone insufficiencies. Since some of these signs actually could be called forth in normal animals by administration of suitable inhibitory structural analogues, it was argued that if one wished to produce certain of these manifestations for a specific purpose, it would be possible to predict the structure of an agent which would do it and thus to postulate a new type of pharmacological agent. That such agents could be predicted and realized was shown by the synthesis of the phenazine analogue of riboflavin (53), of acetylpyridine (56) and finally of the selective drug,  $\alpha$ -tocopherolquinone (57). This last compound called forth in pregnant mice manifestations similar to those seen in vitamin E deficiency but was without detectable action in nonpregnant animals. The difficulty with this procedure is that many of the signs of deficiency may have no desirability but it may be that for specific purposes some of them will be useful. The history of pharmacology has shown that new types of drugs have been discovered by accident, and that synthesis of related compounds has occasionally led to the development of more active preparations. In the future it may be possible to predict the first member of the series rather than to rely on accident for its introduction, because it may be desirable to produce some pharmacological effect hitherto achieved only by dietary or glandular deficiency. Naturally, this manner of attack is limited at the outset by the number of specific pharmacological effects thus far ascribable to deficiencies.

The competitive antagonism between structurally related compounds, and the possession of striking pharmacological properties by some metabolite analogues, have led to attempts to explain drug action as a manifestation of the competition of the drug with some metabolite in the cell. This view of mechanism has been in existence for many years, and the reviewer can do no better than to refer the reader to the discussion by Clark (3) which antedates by several years the observations on *p*-amino-benzoic acid and the sulfonamides (49). A more adequate hypothesis cannot be advanced today.

However, the data which have accrued since 1937 enable us to attempt an understanding of some riddles of the relationship of structure to biological action. Two phenomena will be probed briefly, more in the hope that the ideas will illustrate the fact that the competition hypothesis is entering into the thinking about drug action, than that they represent the ultimate explanation.

Frequently, compounds unrelated in structure show similar pharmaco-



logical action, and this has been most disillusioning to attempts at rational and orderly explanation. Now, it is known that three structurally different compounds, one a phenazine, and the two others isoalloxazines (7, 20, 53), produce similar results, that is, they bring about reversible signs of riboflavin deficiency. All three are analogues of riboflavin. Thus the reason that these three more or less dissimilar compounds have somewhat similar biological effects might be their analogy to the one metabolite. Another example in which the differences in structure are more pronounced is the case of analogues of vitamin K. Four types of compounds, one a coumarin (33), one a phenazine (27), one a naphthoquinone (58), and one a benzoquinone (57), have been observed to cause signs more or less similar to those of vitamin K deficiency which are competitively reversed by the vitamin. While the four compounds are quite dissimilar among themselves structurally, they are united by their analogy to vitamin K.

Another puzzle has been that two compounds closely related structurally have often been found to possess quite different actions. One would expect that allied substances should have similar biological, or at least pharmacological, effects. Take, for example, the phenazine analogue of riboflavin (53) and iodinin, the phenazine oxide relative of vitamin K (27). Both these agents are phenazines which are bacteriostatic. However, one has been shown to act in competition with riboflavin, and the other with vitamin K. The two structurally similar compounds therefore have fundamentally different modes of action.

## VII. Applications to Enzymology

Since many metabolites are intimately concerned with enzyme reactions, substances which compete with these metabolites have been of interest to enzymologists. The very idea of competitive inhibition between structurally related compounds found its beginnings in enzymology. It has been shown that some of the inhibitory structural analogues of nicotinic acid (9), thiamin (39), and pantothenic acid (28) do inhibit the reaction of enzyme systems which involve these vitamins. These inhibitions were not always specific in that substances unrelated to the analogues might be active in causing interference and, more particularly, in that compounds other than the vitamins reversed the inhibition in some cases.

The differentiation of new enzymic processes has frequently depended on the use of selective inhibitors, *e. g.*, cyanide and fluoride. It may be that metabolite analogues which compete with biologically active com-

pounds will be of aid in this analysis (60). McIlwain by the use of thio-panic acid (pantoyltaurine) has made beginnings in this direction (28). He showed that the utilization of pantothenic acid by resting bacterial cells was an enzymic process and that it was inhibited by thiopanic acid. Furthermore, by the use of pyrithiamin Woolley (54) was able to show the existence in certain microorganisms of a new enzyme system which split this inhibitory analogue. This system was absent from species susceptible to pyrithiamin. Since the susceptible organisms were those unable to synthesize thiamin, the presence of the enzyme seemed to be correlated with the ability of the organism to elaborate the vitamin.

The coexistence in organisms of structurally related pairs of compounds which compete with each other may have importance in metabolic processes. The resultant biological effect of such a pair of analogous substances will depend on the relative amounts of the two, and hence the performance of one of the pair may be influenced merely by changes in concentration of the other partner. The possibility has been discussed for the porphyrins by Granick and Gilder (11) and for certain sex hormones (14, 60).

### VIII. Comments Concerning Mechanism

The action of inhibitory structural analogues appears to be connected with the production of specific deficiency of the metabolite involved. Where the effect produced is merely the inhibition of growth of a bacterial culture, the only reason for concluding that a specific deficiency is the cause is that the effects are erased by addition of the metabolite. However, when an analogue is given to an animal with resultant production of many of the characteristic signs associated with deficiency of the structurally related metabolite (vitamin or hormone), there is a cogent reason for believing that a deficiency is involved. Because of this, the observations on animals beginning with the work on pyrithiamin (64) have been of importance in formulating current views of mechanism. When the signs are prevented or erased by adequate amounts of the metabolite, the case for deficiency is strengthened. Even in the absence of reversal by the metabolite the appearance of signs characteristically associated with deficiency of the related substance would seem to imply the production of a specific deficiency in some way.

The most popular hypothesis to explain why analogues should exhibit competitive antagonism with the metabolite is that the two compounds vie for space on a portion of an enzyme normally engaged with the metabo-

lite. If the concentration of the inhibitor is great enough in comparison to that of the metabolite, the latter is displaced, and the biological system is thereby deprived of it. This hypothesis cannot be elaborated beyond the point reached by Clark (3) in 1937, and adopted by Woods in 1940 (49). While this postulate is a satisfying one logically, there is little experimental proof that the analogues really do displace metabolites from protein combinations. At the time of Clark's discussion, the case of competition between CO and O<sub>2</sub> for the hemoglobin molecule provided the only direct evidence for displacement of a metabolite by a structural analogue. In the intervening years only one other demonstration has been forthcoming. Dittmer and du Vigneaud (5) showed that when the analogue of biotin, biotin sulfone, was added to the complex formed between biotin and the specific protein antibiotin (or avidin), the vitamin was liberated. This demonstration lends considerable support, and would be a convincing argument for the displacement hypothesis if antibiotin were known to be of general metabolic significance. In this connection the finding of antibiotin-like proteins in fish and mammals is noteworthy (62).

Although the foregoing postulate is in harmony with most of the data amassed thus far, and although it is accepted in view of the lack of a better one, some facts are difficult to reconcile with it. Let us examine one of these. With several bacteriostatic metabolite analogues, *e. g.*, sulfonamides (13), pyriithiamin (65), and benzimidazole (52), subinhibitory amounts of the agents produce stimulation rather than inhibition of growth. If the analogue merely displaces the metabolite, why should small amounts have this stimulatory effect?

In raising questions of this character it must be borne in mind that the unexplained observations were made on intact, living organisms. There may be mitigating side reactions which would be sufficient to explain the discrepancies. It would seem that the study of competition between structurally related compounds is of sufficient interest that the basic experimental facts should not become too shrouded in working hypotheses.

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# ADENOSINETRIPHOSPHATASE PROPERTIES OF MYOSIN

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## I. Introduction

The hydrolytic splitting of adenosine triphosphate (ATP) undoubtedly occupies a special position among the numerous reactions which go on in

muscle and which may be put in definite relation to the physiological activity of the tissue. It is the only reaction that has proved indispensable to muscular activity. The whole multitude of chemical changes which constitute glycolysis and respiration can be completely excluded, and yet muscular contractions will go on as long as ATP is available (preformed and regenerated at the expense of phosphocreatine). Since it can be experimentally demonstrated that the cleavage of ATP is able, as the sole exergonic process, to maintain muscular activity under artificial conditions, it is only reasonable to assume that, under normal conditions as well, this very reaction is the immediate source of chemical energy for the work of the muscle.

ATP is the last link in the long chain of reactions which make available, for the performance of mechanical work, the chemical energy inclosed in the carbohydrate molecule. The principal agent that performs this work is the protein, myosin, which constitutes by far the predominant part of the solid structure of muscle fiber and which is unanimously regarded as the contractile substance of muscle.

Already, on purely *a priori* grounds, it should be expected that interaction should occur between the bearer of chemical energy and the moving device of the machine in order that the potential chemical energy should be directly, without intermediate degradation to heat, transformed into mechanical work. It is this idea that is expressed in the words of Meyerhof (41):

“Bei allen Maschinen, die keine Wärmemaschinen sind, muss der energieliefernde chemische Vorgang in die Struktur der Maschine eingreifen können, um an ihr die arbeit-liefernde Veränderung herbeizuführen.”

It is astonishing that this idea did not incite any attempt at experimental proof until unexpected indications of an interaction between the chemical and the mechanical factors of muscle appeared as a result of purely enzymological studies. These studies have shown that myosin possesses the properties of the enzyme, adenosinetriphosphatase, which catalyzes the hydrolysis of ATP and liberates the energy on which the work of the muscle depends.

Myosin can be compared with the piston, and ATP, with the explosive mixture, of a combustion engine. The ingenuity of nature consists in providing the piston with the properties of the ignition plug as well. This analogy is, of course, imperfect. It does not reflect one very important point. In the combustion engine, the chemical reaction, once started, proceeds in the main independently of the igniting device, and it is the movements of heated molecules that exert pressure on the piston and bring

it into motion. In muscle, the bearer of chemical energy acts immediately on the moving mechanism, and the potential energy is transformed, not into the thermal movements of molecules, but into forces of intermolecular or intramolecular attraction.

A very remarkable feature of myosin as an enzymic agent lies in the fact that not only does the catalyst produce chemical change in the substrate, but the substrate in turn acts upon the catalyst and changes its physical properties. In what follows, it is intended to give an outline of both these aspects of myosin behavior. The excellent review by Bailey (2) gives a comprehensive survey of the general properties of myosin, and the reader is referred to this article for information concerning the physical and chemical characteristics of the protein.

### *Nomenclature*

It may be stated beforehand that the enzymic activity found in myosin is specifically limited to the hydrolytic splitting off of the third phosphate residue in adenosine triphosphate (ATP), the pyrophosphate linkage in adenosine diphosphate (ADP) remaining untouched by the enzyme. The enzyme is adenosinetriphosphatase and will be designated throughout this text as ATPase. An enzyme which splits specifically adenosine diphosphate only, without being able to attack adenosine triphosphate, would be designated as ADPase; as will be shown later, the existence of such an enzyme has not yet been sufficiently demonstrated and appears questionable. For an enzyme that would split both pyrophosphate linkages without specific discrimination between the di- or triphosphorylated nucleotide, the name "apyrase" has been coined by Meyerhof (43); the liver enzyme, studied by Jacobsen (30) and the enzyme of potato, described by Kalckar (32), are representatives of this kind. The designation "pyrophosphatase" is reserved for the enzyme that attacks inorganic pyrophosphate only, without acting on polyphosphorylated nucleotidic compounds. For the enzyme which splits the ester linkage in adenylic acid, the name "adenylase" is proposed. Finally, there are the "phosphopherases"—the variegated group of enzymes concerned with transphosphorylations between adenylic compounds and different acceptors and donators; but these enzymes will not occupy us here.

## **II. Discovery of ATPase Properties of Myosin**

It is well known that when muscle tissue is crushed, its ATP content rapidly decreases. The disappearance of ATP is accompanied by a roughly corresponding increase of orthophosphate. This clearly indicates the presence of an active ATPase in muscle tissue. Evidently, the important role of this enzyme has not been sufficiently appreciated, for during an astonishingly long period no systematic study of muscle ATPase appeared. This must have been partly due to the fact that, at that time, the attention of all workers in the field of muscle enzymology was almost exclusively di-



rected toward the study of those enzymes which pass over into the aqueous or dilute saline extract of muscle tissue. Here the whole diversity of catalysts participating in the glycolytic cycle can be detected and conveniently studied. But ATPase is often practically absent; probably this was the reason that this enzyme remained neglected for such a long period of time. Only in the paper of Lohmann (37) has it been shown that in crayfish muscle the process of splitting ATP is limited to the first phosphate residue only; the liberation of the second phosphate residue can be induced by the addition of magnesium ions. This led Lohmann to the conclusion that two distinct enzymes accomplish the cleavage. A similar mechanism was postulated for vertebrate muscle, although without direct experimental evidence. No attempts have been made to isolate the enzyme, or even to bring the enzymic activity into solution.

When a study of ATPase of vertebrate (rabbit) muscle was undertaken, it became at once evident that the enzymic activity was to be sought in the insoluble part of the tissue remaining after the preparation of the commonly used aqueous extract. It has been the practice to regard this material merely as a "residue," chemically inert and useless. This attitude must definitely be abandoned. The structural proteins, representing the working parts of the living machine, may have further unexpected chemical activities important for the functioning of muscle. As will be shown in a later section, recent research has brought new evidence to support this statement.

As already stated, aqueous extracts of muscle possess scarcely any ATPase activity. The activity originally found in the unfractionated minced tissue can be almost quantitatively demonstrated in the solid fraction, and remains there without significant losses even after repeated washings with water. When the washed residue is treated with slightly alkaline salt solution of sufficient strength, as is usually employed for the extraction of myosin (*e. g.*, 0.6 *M* potassium chloride with 0.01 *M* sodium carbonate and 0.04 *M* sodium bicarbonate) as proposed by Weber (72), the activity is found to go almost completely into solution, and from now on the activity accompanies the fraction of myosin, whatever further attempts of fractionation are applied. The question of the actual identity of ATPase with myosin will be discussed later when a survey of the properties of the enzyme is given.

### III. Purification of ATPase

No very elaborate methods have been as yet applied to the purification of ATPase. Serious difficulties arise from the very great lability of the

enzyme. Since the enzymic activity is found in the myosin fraction, no specific method for the isolation of the enzyme could have been devised up to the present time, save those proposed for the preparation of myosin.

The usual method is to extract the minced tissue with ice-cooled, rather concentrated salt solutions (0.5–0.6 *M* potassium chloride or lithium chloride) buffered to a *pH* of 8.5–9 for 20–30 min. with continuous stirring and then to filter the fluid through paper pulp. Dilution of the extract with 10–20 volumes of iced water precipitates the myosin, and the enzymic activity is found in the myosin sediment. The latter is dissolved in a small volume of water by addition of salt, filtered, and reprecipitated in the same manner. Several other enzymic activities besides that of ATPase can be found in the first myosin precipitate prepared from minced muscle not previously washed out with water, but on reprecipitation they are rapidly eliminated and a solution of thrice precipitated myosin appears enzymically homogeneous.

### 1. Crystalline Myosin

A substantial advance in the study of myosin was made by Szent-Györgyi, who succeeded in obtaining myosin in crystalline form.

It had been noticed by previous investigators (2) that at certain stages in the precipitation of myosin by dilution, a pronounced silklike sheen appeared on stirring the mixture, a phenomenon commonly known to indicate the formation of needle-shaped microcrystals. But myosin had always been regarded as a typical representative of fibrous proteins, which have never been known to be crystallizable, so that the idea evidently never occurred to anyone that it could be obtained in crystalline form; and the observation was left without due consideration. It remained for Szent-Györgyi\* to work out a method which is stated consistently to give crystalline preparations of myosin that can further be repeatedly recrystallized (64, 66).

The principle of the method consists in the removal of a second protein, "actin," present in the crude extract and forming with myosin, a compound called "actomyosin."

**First Step.**—Minced muscle is extracted for ten minutes with constant stirring and three volumes of an ice-cold mixture of 0.3 *M* potassium chloride and 0.15 *M* potassium phosphate of *pH* 6.5. The mass is centrifuged or strained through cloth. Four volumes of water at room temperature are added for each volume of chloride-phosphate, and the mixture is rapidly strained through cloth and stirred gently for an hour or two until a flocculent precipitate of actomyosin is formed. The precipitation is caused by a decrease in the concentration of ATP, originally present in the extract and slowly split

\* The separate papers representing the recent research of Szent-Györgyi and his collaborators were published in three successive issues (63–65) of *Studies from the Institute of Medical Chemistry, University Szeged*, which appeared between 1941 and 1944. The material has been summarized by Szent-Györgyi in monograph form (66). Compare also A. Szent-Györgyi, *J. Colloid Sci.*, **1**, 1 (1946).

during incubation. Whereas higher concentrations of ATP dissolve actomyosin, low concentrations precipitate the compound. If the fluid is left without stirring, the precipitate is colloidal and cannot be separated in the centrifuge.



A



B

Fig. 1.—Myosin crystals, clusters, and microscopic threads (66). Magnification: A,  $\times 90$ ; B,  $\times 40$ .

The precipitate is separated by rapid centrifugation at room temperature, and the opalescent fluid is diluted with 1.5 volumes of ice-cold water run in slowly in about ten minutes under constant, energetic stirring. The myosin separates in the form of fine,

needle-shaped crystals. If the water is added at once and without stirring, an amorphous precipitate is obtained. The mixture is allowed to stand for an hour or two at 0°, decanted, the myosin separated in the centrifuge, and washed by suspending it in 0.04 *M* potassium chloride and centrifuging.

**Second Step.**—The crystalline myosin precipitate of the first step is dissolved in a 0.02 *M* potassium carbonate solution containing 0.01% phenolphthalein. The carbonate solution is added in small quantities, with strong stirring, until a faint rose color persists (pH 8.3). Then 4 ml. of 2 *M* potassium chloride is added for every gram of myosin present. The mixture is diluted with water, of which 50 ml. is added for each milliliter of potassium chloride. The water is at room temperature (22°) and contains 0.001% phenolphthalein and sufficient potassium carbonate to give it a faint rose color. The water is added while stirring vigorously. The voluminous, loose precipitate formed is separated in the centrifuge and the slightly rose colored, opalescent supernatant fluid is decanted and cooled. The remaining precipitate is again treated in the same way as previously; if the rose color has vanished, potassium carbonate is added until the color reappears; potassium chloride is added and the whole is diluted with water, the only difference being that this time only half as much potassium chloride and water are used. The precipitate is centrifuged and discarded, and the fluid mixed with the one obtained previously. From this point on, the procedure is continued at 0°.

The fluid is stirred vigorously, and 1% acetic acid is run in very slowly until the fluid is neutralized to pH 7. The myosin separates in the form of somewhat irregular needles and is centrifuged.

**Recrystallization.**—The precipitate is dissolved by adding 2 *M* potassium chloride in small quantities, until a concentration of 0.6 *M* is attained. The fluid is carefully homogenized after each addition. Then the mixture is further diluted with 0.6 *M* potassium chloride until it loses its very high viscosity and contains about 3% myosin.

The myosin solution is stirred vigorously and water is run in very slowly until the potassium chloride concentration becomes 0.04 molar. The addition of this amount of water should take about one hour. The myosin separates in the form of beautiful needle-shaped crystals. About half of the myosin isolated in the first step will be obtained in this form.

The preparation of crystalline myosin involves considerable losses. The total yield of crystalline material is about 10% of that originally extracted from muscle.

Recrystallized myosin is reported by Szent-Györgyi to contain 3% of the dry weight of lipid matter [ordinary preparations of myosin contain 5–10% of lipid matter—see Bailey (1)]. The most remarkable property of crystallized myosin appears to be its solubility. The following is the description given by Szent-Györgyi (64):

If the crystalline myosin is suspended in water and is rendered salt-free by dialysis it swells up to a glassy mass which shows only a slight opalescence. On dilution it dissolves in water giving a clear and very viscous solution from which no myosin can be separated by centrifugation. According to the water solubility in complete absence of salts, myosin is not a globulin. On the other hand, on adding ammonium sulfate the myosin precipitates as we pass half-saturation. In this respect myosin conforms with globulins.

Myosin is precipitated by small concentrations of neutral salts and dissolves in the presence of higher salt concentrations. If dissolved in water or in 0.1–0.2 *M* potassium chloride it shows a very strong flow birefringence. The flow birefringence becomes weaker at 0.3 *M* potassium chloride and disappears entirely at 0.4 *M* potassium chloride. It also disappears if the solution is rendered alkaline.

When ATP is added to an aqueous solution of crystallized myosin, the viscosity decreases and the flow birefringence becomes somewhat weaker.

Table I, taken from a paper of Szent-Györgyi (64), shows the action of different salts on crystallized myosin.

TABLE I  
EFFECT OF DIFFERENT SALTS ON THE SOLUBILITY OF CRYSTALLIZED MYOSIN (64)\*

Salt	Final molarity									
	0.2	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	0.0008	0.0004
KCl	0	0	+	++	++	++	+	+	0	0
KF	+	+	+	++	++	++	++	++	+	0
KI	0	0	+	++	++	+	0	0	0	0
LiCl	0	0	+	+	++	++	++	+	+	0
NaCl	0	+	+	++	++	++	++	+	0	0
MgCl <sub>2</sub>	0	++	++	++	++	++	++	++	++	++
CaCl <sub>2</sub>	0	++	++	++	++	++	++	++	++	++

\* 0.5 ml. of the salt solution added to 2 ml. of 0.1% salt-free myosin solution. 0, no change; +, turbidity or precipitation.

This behavior of crystallized myosin insofar as solubility is concerned is quite unique. There are no analogies in all that is known about other proteins. That the observed phenomena are changes of solubility in the strict sense of the word, however, is not sufficiently evident. From observations of Edsall (17), it is known that ordinary myosin precipitates show a remarkable tendency to swell up in water in the absence of salts to a glassy mass having the properties of a thixotropic gel. In this respect, there is no difference with what is obtained with crystalline myosin when the material is freed of salts by dialysis. The difference—and a most important one—appears when it is stated that, on the addition of water, the swollen gel, if sufficiently intimately mixed, goes into solution from which no material can be separated by centrifuging. Now a reliable criterion of true solution is the ability to obtain equilibrium mixtures consisting of excess of soluble phase and saturated solution. This criterion can be readily applied to the solution of a myosin gel in salt solutions of different concentrations and at different *pH* values (60). Nothing of the kind has been reported for crystalline myosin “dissolved” in water. Until evidence of this kind is obtained, it would be safer to interpret the phenomena observed as being

caused, not by a solution, but by a further swelling of the gel. The effect of small concentrations of salt would be to inhibit the swelling and to produce turbidity. There is no doubt that final decisions to questions touching upon the fundamental properties of proteins can hardly be based upon purely qualitative observations. Detailed quantitative experiments are urgently needed before any definite conclusion can be drawn.

The recrystallized myosin possesses the ATPase activity observed in ordinary preparations. According to the data of Banga (5), crystallization does not produce any marked increase of the enzymic activity of myosin. This is not astonishing because the purification achieved by crystallization consists chiefly in the removal of the whole actin, originally present in the crude myosin preparation, and of about half of the lipid impurities. The admixture of actin in the crude preparations of myosin obtained by short extraction is estimated by Szent-Györgyi to be about 1% of the amount of myosin; the decrease in lipid matter corresponds to about 3-5% of the weight of myosin. So the process of crystallization would result in the removal of admixtures, which in the usual, noncrystallized preparations of myosin total not more than a few per cent. Such differences would not perceptibly influence the values of the enzymic activity, the precision of the determinations not being very great.

For the present at least, the fact that crystallization of myosin has been achieved would seem to be more important for the study of the physical properties of myosin than for the investigation of its enzymic properties.

## 2. Soluble ATPase

It should be mentioned that, according to observations of Sakov (57) and of Needham (47, 48), ATP can also be attacked to a slight degree by the soluble fraction of muscle tissue. The properties of the observed enzymic activity differ widely from those of myosin (see Table II).

TABLE II  
ENZYMIC PROPERTIES OF THE SOLUBLE FRACTION AND OF MYOSIN (57)

Properties	Soluble fraction	Myosin
Solubility	Goes over in aqueous extract	Not extracted from muscle by water
Mode of action	Splits off two phosphate residues	Splits off one phosphate residue
pH optimum	About pH 6.0	About pH 9.0
Heat inactivation	0% in 30 min. at 37° 33% in 30 min. at 45° 90% in 30 min. at 50°	60% in 5 min. at 37° 100% in 10-15 min. at 37°

When referred to the same amount of tissue, the activity of the soluble fraction is only a few per cent of the activity of myosin (47). The role of this soluble enzyme is probably to be regarded as that of a release valve, serving to regenerate the phosphate during periods when there is no need for the participation of myosin.

### 3. *Actin*

New contributions of unquestionable significance for the future development of muscle chemistry have been made during recent years by Szent-Györgyi and his collaborators. The preparation of crystalline myosin has already been mentioned. Another important discovery was that of a new protein—actin (61, 62). Like myosin, it belongs to the group of insoluble proteins, being even less soluble than myosin. When myosin is extracted from muscle tissue, actin remains in the residue and can be obtained from it after the residue has been treated with acetone.

The discovery of the ATPase activity of myosin and the discovery of actin have a feature in common. In the former case, the residue—usually thrown away after the extraction of the soluble constituents of muscle tissue—had to be salvaged from the discard in order to provide the bearer of ATPase properties. In the latter case, Szent-Györgyi and his group made use of the residue—usually thrown away after the extraction of myosin—and discovered a new protein having remarkable properties.

*Preparation of actin* (after Straub, 62). Muscle is cooled and finely minced; 300 ml. of an alkaline potassium chloride solution\* is added to every 100 g. of tissue and the mass is stirred 20 min. at 0°, and then centrifuged. The supernatant material is discarded and the residue is left at 0° for 24 hrs. It is weighed and mixed with five volumes of its weight of distilled water at room temperature, left to stand for 1 hr., and centrifuged. The washing is then repeated as previously. After this second washing, the residue is treated with 4 volumes of acetone at room temperature. After standing 20 min. the acetone is removed by pressing it out through a cloth. The residue is mixed with a fresh lot of acetone (one-fourth of the former volume) and, after standing 20 min., is pressed out, spread on filter paper, and left to dry.

The acetone-dried muscle powder (after 10–15 hrs. drying) is extracted with twenty volumes of carbon dioxide-free water at room temperature by thorough mixing and then standing for 10–15 min. The fluid is sucked off by a Büchner funnel. The protein content of this extract varies between 3 and 6 mg. per ml. It contains actin in a high state of purity. On the average, the actin content is about 5 mg. per ml.

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\* 800 ml. 0.1 *M* potassium borate is mixed with 200 ml. of 2 *M* potassium chloride. The potassium borate solution is prepared by dissolving 12.4 g. boric acid in 100 ml. *M* potassium chloride and then the solution is made up to 1 liter.

The properties of actin, described in great detail in the papers of Straub (61, 62), can be sketched here only briefly, mention being made of points to be referred to in discussing the enzymic properties of myosin.

Actin can be obtained from muscle in an "inactive" form. Its solutions have low viscosities, which is to be expected, and no flow birefringence is observed. Actin in this state is regarded as being in the globular form and is designated as "G-actin," "G" standing for globular. Small amounts of salts produce a transformation of G-actin: the fluid becomes opalescent, and exhibits a very great and highly anomalous viscosity and a strong flow birefringence, which persist for some time after stirring. The protein in this form is thixotropic; a 0.4% solution gelatinizes on standing. It is assumed that these changes result from a transformation of the globular protein into a fibrous one. The new form is designated as "F-actin"—"F" representing fibrous. The viscosity of F-actin is not influenced by ATP. The F-G transformation is reversible: if the salt that has transformed G- into F-actin is removed by dialysis, the properties of G-actin reappear. F-actin reacts with myosin to form a highly viscous actomyosin. The F-G transformation is accelerated not only by ions, but, also, at definite salt concentrations, by myosin.

The amount of actin in muscle is considerable. Szent-Györgyi (66) states:

According to Balenović and Straub [3] 1 g. rabbit muscle contains 25–30 mg. of actin, representing about 12–15% of the total protein. It is evident that the insoluble "stroma," which makes up about 15–20% of the total protein of muscle, must consist chiefly of actin. If blood vessels and connective tissue are discounted, no room is left for any other protein to play a significant quantitative role in the composition of muscle.

It is astonishing that actin, which must be regarded as the principal constituent of the insoluble muscle stroma, is readily extracted by water from the acetone-treated residue and when extracted appears to be easily soluble. No explanation is given by the authors for this strange fact. It seems probable that actin is present in the tissue as a lipoprotein, a compound of the protein and some lipid, and that it is the lipid which makes the protein insoluble. Acetone splits the complex, removes the lipid, and actin becomes soluble. It would be interesting to determine whether the acetone extract can again change the properties of extracted actin.

#### 4. *Actomyosin*

When dilute solutions of F-actin and myosin are mixed, a great rise in viscosity is observed, the value found being more than twice the sum of the viscosities of the two substances. The increase in viscosity is attributed



to the formation of a compound of F-actin and myosin, actomyosin; the decrease in viscosity, produced by ATP, is attributed to a dissociation of this compound into its components, actin and myosin. This decrease in viscosity produced by ATP provides a measure of the quantity of actomyosin. The maximum effect is obtained when the actomyosin contains two parts of actin to five parts of myosin; it is approximately in this ratio that both substances are present in muscle.

Some properties of the components are found unchanged in the actomyosin, others are modified. The ATPase activity of myosin remains quantitatively unchanged when myosin combines with actin to form actomyosin, and the activating effect of calcium is the same in both cases. But the behavior toward magnesium is altered: whereas magnesium inhibits the activity of myosin ATPase, the ion has no inhibitory effect on the enzymic activity of actomyosin.

#### IV. Characteristics of Enzyme Properties of Myosin

##### 1. *Specificity*

In discussing the specificity of the enzymic properties of myosin, we must consider the specificity of action and the substrate specificity.

As has already been mentioned, the enzymic action of myosin as ATPase is strictly specific in that only one of the two pyrophosphate bonds present in ATP is split, the second being left untouched. With excess of myosin and sufficient duration of incubation, exactly one-half of the 7'-P is liberated and adenosine diphosphate (ADP) is found as the end product.

Lohmann (37) was the first to observe, in experiments with washed crayfish muscle, the partial dephosphorylation of ATP, with the splitting off of one phosphate and the formation of ADP. The latter can subsequently be hydrolyzed further by the action of other enzymic agents activated by magnesium ions. A similar two-step mechanism of hydrolysis of ATP, with the participation of different enzymes, was postulated for vertebrate muscle as well, and the results obtained with myosin prove the correctness of this view.

The fact that the action of myosin on ATP is limited to the liberation of only one phosphate residue was discovered early (21, 38) and was confirmed by all subsequent investigations (1, 5, 47). When thrice reprecipitated myosin is used, the ADP formed is not attacked further and remains as an end product that can be isolated. This permitted Lyubimova and Pevzner (39) to propose a convenient method of preparing ADP: the use of repre-

cipitated rabbit myosin as the dephosphorylating agent instead of washed crayfish muscle used by Lohmann (37).

The substrate specificity of myosin appears to be marked but not absolute. Hexose diphosphate and  $\alpha$ -glycerophosphate are not split by myosin (47) and diphenyl pyrophosphate and inorganic pyrophosphate were also found by Bailey (1) not to be attacked by myosin. Nevertheless, ATP is not the only substrate upon which the enzyme can act. In order that a substance may serve as substrate for the enzymic action of myosin, a necessary condition is the presence of the triphosphate grouping. The structure, and perhaps even the very presence, of the nucleosidic grouping influence the substrate properties only quantitatively—not in a qualitative manner. Kleinzeller (33) observed that myosin readily splits the deaminated derivative of ATP—inosine triphosphate. The reaction rate is about three times higher than with ATP. Here again only one phosphate residue is split off, inosine diphosphate appearing as the end product. It is possible that an even greater extension of the range of specificity ascribed to myosin will become necessary, as indicated by the results reported by Needham and co-workers (12, 49). Extending the observations of Neuberg and Fischer (50), that inorganic triphosphate is hydrolyzed by kidney and muscle tissue, the Cambridge authors investigated the behavior of myosin toward this substance. The triphosphate was found to be split by myosin preparations. The rate of hydrolysis was several times slower than with ATP, but still considerable. Calcium ions, known as necessary activators of ATPase, accelerate the hydrolysis of triphosphate as well. Noteworthy was the final result: not more than about 29% of the total phosphorus appeared as orthophosphate. It may therefore be assumed that the splitting of the inorganic triphosphate proceeds in the same way as the breakdown of the natural nucleotidic compound, one phosphate being liberated and the two others remaining as pyrophosphate. This would be in accordance with the (already mentioned) inability of myosin to hydrolyze inorganic pyrophosphate.

There is still some doubt as to whether the splitting of inorganic triphosphate is actually caused by myosin itself and not by a mixture of myosin and another enzyme. Large quantities of myosin preparations have been used in the experiments reported. With smaller quantities, Bailey could not observe any splitting of inorganic triphosphate under conditions in which ATP was readily attacked.

However, evidence of quite a different kind also indicates the existence of specific relations between myosin and inorganic triphosphate. Valuable information about the specific affinities of an enzyme can often be obtained

from the study of competitive inhibition. Figure 2, plotted from the data of Dainty *et al.* (12), demonstrates clearly the strong inhibitory effect of inorganic triphosphate on the splitting of ATP. Evidently the triphosphate possesses an affinity for the same grouping in myosin that reacts with ATP. It can be expected that triphosphate, being able to combine with ATPase, is also acted upon by this very enzyme, and is split in the same way as is ATP.

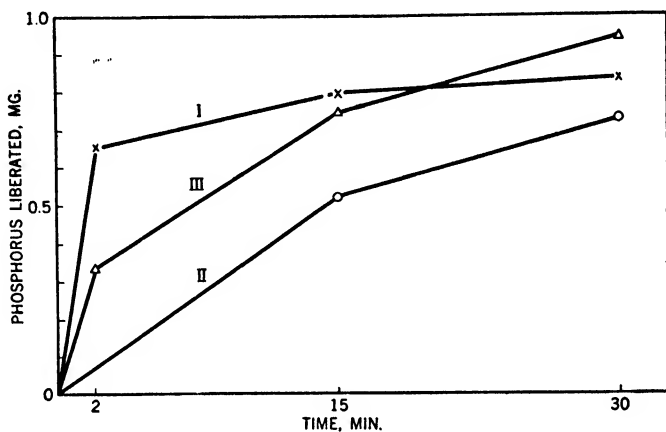
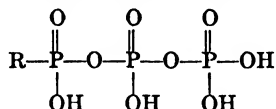


Fig. 2.—Competitive inhibition of ATPase activity of myosin by inorganic triphosphate (12). Curve I, myosin (1.48%) acting on 0.01 *M* ATP; II, myosin (1.48%), on inorganic triphosphate (0.015 *M*  $\text{Na}_2\text{P}_2\text{O}_7$ ); III, myosin (1.48%), on 0.01 *M* ATP plus inorganic triphosphate (0.03 *M*  $\text{Na}_2\text{P}_2\text{O}_7$ ).

And, finally, one may interpret in a similar manner the results of experiments of a more indirect nature, where not the enzymic activity but physical changes of myosin were investigated. ATP greatly reduces the flow birefringence of myosin solutions. Inorganic triphosphate, which has no such action, diminishes the effect of ATP by 20 to 70% (12). Of a large number of substances tested, only the triphosphate, and to a smaller degree ADP, had this effect. Evidently, here again we have the result of competitive interaction.

All these facts strongly support the view that myosin itself, and not an occasional admixture with another enzyme, is responsible for the splitting of inorganic triphosphate. In summary, it may be said (12, 49) that the triphosphate structure is the determining condition for the enzymic action of myosin:



The nature of R (OH in the case of triphosphate, adenosine in ATP, inosine in ITP) seems to influence primarily the physical properties of myosin, rather than its enzymic effect.

**Splitting of Adenosine Diphosphate.**—The mechanism of the enzymic breakdown of ADP seems to be much more complicated than was expected. At least three paths of breakdown of ATP to adenylic acid are clearly possible, and perhaps still further complications exist. Formation of adenylic acid from ATP can proceed: (a) by the action of two distinct enzymes, ATPase and ADPase having a rather strict specificity and working in tandem; (b) by the action of one enzyme of lesser specificity (apyrase), able to attack ATP as well as ADP and therefore splitting off both labile phosphate residues from ATP; (c) by the combined action of ATPase and myokinase.

The first reaction was believed to occur in muscle (37, 38), but in light of the recent findings of Kalckar (31) this interpretation must be revised. The presence of a true ADPase in muscle, although not definitely disproved, has not yet been demonstrated, and the effects previously ascribed to a water-soluble ADPase (21) are in all probability produced by reaction (c).

Enzymes acting as in (b)—apyrases—are probably those of liver (30) and potato (32). At least it can be said that here mechanism (c) is excluded, as no myokinase has been found by Kalckar in liver, and the enzyme is obviously absent from the purified preparation of potato. However, for the very crude preparations from liver a two-enzyme mechanism of the type indicated in (a) is still quite possible, since no efforts have been made to establish whether or not the two activities can be separated by fractionation or selective inhibition.

In muscle, according to Kalckar (31), mechanism (c) is at work. Myokinase catalyzes the "dismutation" of the ADP formed by the action of myosin:



The ATP formed is split again by myosin to ADP, and the process proceeds to the exhaustion of the available substrate. Thus, phosphate is liberated from ATP and adenylic acid is formed from ADP, and while ATP disappears, adenylic acid and orthophosphate accumulate. An ADPase effect can easily be simulated when reprecipitated myosin, which by itself does not

attack ADP, is permitted to act on this substance in the presence of added myokinase. Here ADP is dephosphorylated, although the phosphate actually arises from ATP.

Banga (5) claims that there is still another enzyme which is present in muscle and which acts on ADP; it is distinct from myokinase and designated as ADP isomerase. This enzyme is said to produce a change ("isomerization") in ADP, evidenced by the behavior toward myosin. Ordinary ADP is not attacked by myosin or by actin taken separately, or by the complex of both, actomyosin. The "isomerized" ADP, still not attacked by myosin or actin separately, is reported to be split when both proteins are present together, *i. e.*, in the form of actomyosin. As the nature of the postulated "isomerization" remains completely obscure, more detailed and substantial experimental evidence must be awaited before these conclusions can be regarded as being sufficiently well established.

## 2. *Thermolability and Stabilization of ATPase*

Myosin is known to be one of the most heat-sensitive proteins. It is rapidly denatured even at temperatures below 40°. The loss of ATPase activity seems to be one of the first changes observable in myosin solutions after only exposure to the action of moderate heat. At 37° the activity is greatly reduced within 5 min. and is almost completely lost in some 10 to 20 min. The question naturally arises as to how myosin exists in the warmblooded animal. It is evident that conditions must exist which stabilize the labile protein against thermal denaturation and inactivation.

One such factor is the ATP itself; in its presence the stability of myosin is found to be great. It is a common experience that naturally occurring conjugated proteins are very stable so long as they are combined with the corresponding apoteon,\* but become very labile as soon as they are separated from the latter. Perhaps the best example of this kind is globin, extremely stable in the proteid form as hemoglobin, and most readily undergoing spontaneous denaturation as soon as it is separated from hemin. Another case is that of the old "yellow enzyme," the protein of which is much more easily denatured when it is separated from the flavin moiety (68).

The stabilizing action of ATP makes it evident that myosin readily combines with the nucleotide and that the ATP-myosin conjugate so formed is much more stable than free, unconjugated myosin. The assumption is perhaps justified that in muscle myosin is present as ATP-myosin complex.

\* The term *apoteon* is suggested to designate the nonprotein moiety of conjugated proteins. It corresponds to the generally accepted *aplycon*.

An attempt has been made by Lyubimova and Shipalov (40) to obtain direct evidence of the formation of such a complex by means of ultraviolet spectrophotometry. As a rule, conjugation is accompanied by a more or less pronounced change of the spectral properties of the apoprotein. The marked changes in the spectrum when hemin becomes bound to globin are a familiar example. Many other substances exhibit the same phenomenon. The absorption maximum of flavin is shifted about 20 m $\mu$  to the red end when the substance becomes conjugated with its specific protein partner (69); also, great changes are observed in the spectra of carotenoids when they form protein complexes. The adenine residue of ATP has a characteristic absorption maximum at about 260 m $\mu$ . It is to be expected that, when ATP becomes conjugated with myosin, a displacement of the absorption band may occur; but in the experiments reported no such shift was observed when ATP was brought into contact with myosin: no difference was observed between the absorption curve of the mixture and the curve obtained when both components were placed in the path of light in two separate cuvettes. Other methods should be tried to demonstrate the formation of the ATP-myosin complex.

Glycine somewhat increases the heat stability of myosin solution (1). The effect, as in the case of increase of activity, might result from the removal of traces of heavy metals which possess a deleterious action on the ATPase.

Still other factors, apart from the action of ATP, could contribute to the stabilization of myosin in the cell. One of them might be the peculiar state in which myosin is present in the fibril, where it is at least ten times less hydrated than in the most concentrated gels that can be obtained outside the cell. The thermolability of myosin in solution seems to increase with dilution; and therefore in the highly concentrated state existing in the fibril the stability could be markedly increased.

Finally, the thermolability of myosin could be influenced by the formation of complexes with other proteins. As already mentioned, profound changes in the physical properties of myosin occur when myosin combines with actin to form actomyosin. It might well be expected that thermostability may also be considerably influenced, although data regarding the relative heat sensitivity of myosin and actomyosin have not yet been reported.

### 3. *pH Dependence*

The maximum activity of ATPase is attained rather far in the alkaline region, at about pH 9 (4, 38). On closer examination, the activity curve

shows a peculiar shape with two maxima, one at pH 6.3, and the other, about twice as high, at pH 8.9–9.1 (22). See Figure 3.

It would seem natural to interpret this very strange character of the pH-activity curve, not found with any other enzyme, as indicating the presence of two distinct enzymes with different pH optima; "acid" and "alkaline" phosphatases are well known among the enzymes that act on the simpler organic esters. But, up to the present, all attempts to separate the two presumable components and to obtain preparations showing only one pH

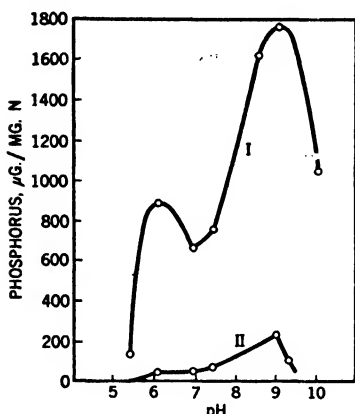


Fig. 3.—pH-activity curve of myosin-ATPase (22). Curve I, ATP as calcium salt; II, with 0.01 *M* magnesium chloride.

optimum have failed. "Acid" and "alkaline" phosphatases are known to be influenced in a very different way by magnesium ions. If two enzymes were present in the myosin preparations, different effects might be expected to result from addition of magnesium in the acid and alkaline regions, but this is not the case: the whole curve is found to be smoothly displaced, magnesium giving a strong inhibitory effect over the whole range between pH 5.5 to 10.

A possible explanation of the peculiar character of the pH-activity curve may be that the activity in this case is determined simultaneously by the ionization of the enzyme, as postulated by Michaelis, and the ionization of the substrate, as suggested by Northrop. The irregular shape of the curve would then be the result of a summation of the two independent variables.

#### 4. Activators and Inhibitors

**Influence of Ions.**—The ATPase activity of myosin is strongly influenced by ions, as is the activity of most of the other enzymes concerned in the metabolism of phosphorus compounds (phosphatases, isomerases, phosphophorases).

In the case of most of these enzymes, magnesium is known to act as a powerful activator, being often replaceable by manganese and sometimes by other ions as well. With ATPase, the conditions are distinctly different. Calcium appears to be an indispensable factor for the activity of the enzyme. Under carefully chosen conditions (purified preparations of myosin, with small quantities being used in the experiments in order to minimize

the effect that might be produced by ions contaminating the protein), the activity found in the absence of added activators is scarcely measurable. Addition of calcium increases the activity about tenfold at least; manganese has a much smaller effect (1, 67).

According to data of Banga (4), maximum activation is obtained with about 0.01 *M* calcium chloride, whereas the experiments of Greville and Lehmann (28) indicate a somewhat smaller figure, about  $3 \times 10^3$ . The degree of activation seems to depend also on the ATP concentration (1).

The effect of magnesium is remarkable—almost the opposite of that observed with other enzymes. Not only has magnesium no activating effect, but, on the contrary, in systems activated by calcium it produces a very strong inhibitory effect (39).

The antagonistic action of calcium and magnesium on the ATPase activity of myosin must be an important regulatory mechanism in the functioning of the muscle cell. DuBois and Potter (16) regard the inhibitory action of magnesium on ATPase as being responsible for the phenomenon of magnesium narcosis. Certain objections to this view are raised by Greville and Lehmann, who are inclined to believe that the region of application of the narcotic effect of magnesium is in the neuromuscular junction rather than in the muscle cell itself.

Activation by calcium is not characteristic of myosin-ATPase only. According to data reported by DuBois and Potter (16), calcium-activated ATPase is found in many different tissues. The only exception known at present seems to be the electric organ of *Torpedo*, in which ATPase, according to Greville and Lehmann (28), exhibits a different behavior, being activated by magnesium and inhibited by calcium ions.

Copper (1, 4, 9) and silver (23) ions inactivate the ATPase completely, even in very low concentrations; silver is especially active, almost complete inactivation being observed even at concentrations of the order of  $10^{-5}$  *M*. It is important to use glass-distilled water in all experiments on the ATPase properties in order to avoid traces of copper, which may greatly influence the results.

The action of ATPase being the principal cause of losses during isolation of ATP from muscle, it has been proposed to make use of the inhibitory effect of silver (22) and of magnesium (15) in order to inactivate the enzyme and so to increase the yields of ATP or to avoid the expenditure of large amounts of deproteinizing reagents.

**Enzyme Poisons.**—Muscle ATPase has been reported (22) to be completely insensitive to cyanide, even in concentrations up to 0.1 molar. Binkley (9) finds that cyanide of this strength exerts a certain inhibitory



effect. Yet with 0.01 *M* cyanide there is not only no inhibition, but even an increase of activity, which may be ascribed either to a removal of inactivating metal ions (copper), or to an increase of sulfhydryl groups. Phlorizin, known to have a strong inhibitory effect on several reactions of phosphate transfer, produces, in concentrations of 0.01–0.02 *M*, a slight inhibition, observable only in the acid region (pH 6.2). Fluoride, which inhibits many hydrolytic phosphatase reactions, has a certain inhibitory effect on ATPase, probably by virtue of removal of calcium ions; the effect is especially pronounced when the enzyme is activated by calcium but otherwise may not result. Perhaps the most interesting results that might shed light on the nature of the chemical groupings involved in the enzymic activity could be expected from a study of the action of iodoacetate, the inhibitory effect of this poison being regarded as a result of interaction with sulfhydryl groups in the enzyme molecule. The results obtained will be dealt with in the next section.

Urea in sufficiently high concentrations splits the myosin particles into fragments of about ten times smaller molecular weight (*ca.* 100,000), according to Weber and Stöver (73). This is accompanied by a change of the optical properties of myosin (disappearance of the flow birefringence) and it is not surprising that the enzymic properties of myosin are also observed to be lost.

**Role of Sulfhydryl Groups.**—In recent years, increasing attention has been paid to the role of thio groups in enzymes. They appear to be essential for the action of a considerable number of enzymes and probably participate in the regulation of enzymic processes. A valuable survey of the problem has been presented by Barron and Singer (6). All enzymes can be divided into two classes: one requiring the presence of sulfhydryl groups for their action, and the other not depending on their presence. Whether an enzyme belongs to one group or the other may be determined by its behavior toward reagents that react with sulfhydryl or disulfide groups.

Iodoacetate is one of these reagents. Sulfhydryl groups which can be detected by the nitroprusside reaction disappear after treatment with iodoacetate. This is regarded not as a result of oxidation of sulfhydryl to disulfide but as a result of the substitution of the hydrogen by the  $-\text{CH}_2-\text{COOH}$  group. In experiments of the writer (22), a moderate inhibition of ATPase of about 30% was observed when rather high concentrations of iodoacetate (0.01 *M*) were used. Needham (47), using somewhat lower concentrations (0.0033 *M*), found no decrease in the ATPase activity of myosin, although the nitroprusside test became negative. The same result

was obtained with iodoacetamide, which is an even stronger reagent for sulfhydryl groups, and also after treatment with 2 *N* ammonium chloride, which destroys the thio groups.

These experiments do not necessarily establish that sulfhydryl groups are of little or no importance to the enzymic activity of myosin. As in the study of sulfhydryl groups in proteins in general, it is dangerous to rely too much on results obtained by any single test, *e. g.*, the nitroprusside reaction. This is clearly shown in the present case by the significant observations reported by Ziff (74). Myosin solutions, when left in contact with air, show a gradual decrease of ATPase activity. When such preparations are treated with reducing agents, such as cysteine, glutathione, or to a lesser extent, ascorbic acid, which are all known to reduce oxidized sulfhydryl groups, a considerable increase of the ATPase activity is found. Direct oxidation effected by hydrogen peroxide added to a fresh myosin solution decreases the enzymic activity by more than 70%. Here, again, treatment with cysteine or SH-glutathione or with cyanide (9) restores the full activity. Direct determinations of sulfhydryl groups have not been reported for these experiments, but as no other groups in the protein molecule except the disulfide groupings are known to be reduced by the reagents employed in the experiments of Ziff and of Binkley, it may be concluded, with reasonable confidence, that the inactivation and reactivation of myosin-ATPase result from the disappearance and restitution of sulfhydryl groups.

This conclusion is substantially corroborated by the findings of Barron and Singer (7). They have shown that chloromercuribenzoate, a most powerful and specific reagent for sulfhydryl groups, completely inactivates the ATPase function of myosin; subsequent treatment with glutathione completely restores the original activity. Thus, it may be regarded as definitely established that myosin, as ATPase, belongs to the sulfhydryl-conditioned group of enzymes. The discrepancy between the observations just mentioned and the results obtained with iodoacetate is readily explained by the fact that iodoacetate is known to react only with a limited number of the sulfhydryl groups actually present in a protein molecule; evidently these groups are of less importance to the enzymic activity, whereas the groups attacked by oxidative agents, or by the mercuric compound, are essential to the activity of the enzyme.

Finally, mention should be made of the views advanced by Gol'dshtein (26), who has brought forward certain evidence indicating the possibility of thioether linkages of the cystathionine type being present in the myosin molecule. According to Gol'dshtein, these linkages may perhaps participate

in the reaction with ATP, undergoing a splitting in the manner discovered by Binkley (8); this fission of bonds in which sulfur participates may be responsible for the changes in the physical properties of myosin which are produced by the action of ATP.

### 5. Activity Values

The activity of ATPase can be expressed in micrograms of phosphorus liberated per milligram of nitrogen and unit of time. In order to permit a direct comparison of the activity of ATPase with that of other enzymes, it is convenient to express the activity in terms of  $Q_P$ , i. e., the amount of phosphorus expressed as microliters of gas, liberated per milligram of substance per hour (21, 38).

Considering the great lability of the enzyme, a test of short duration (5 min.) is preferable. The quantities of ATP and of the enzyme must be such that not more than about 25–30% of the initial 7'-phosphorus are liberated during the test, leaving a certain amount of available substrate (which corresponds initially to 50% of the 7'-phosphorus) still present at the end. The weight of the enzyme is found by means of the Kjeldahl nitrogen determination, the factor 6.0 being used to translate the nitrogen values into protein (1). One microgram of phosphorus corresponds to  $22.4/31 = 0.7$  microliter of gas, and for a test of five minutes, the value of  $Q_P$  is given by the expression:

$$Q_P = \frac{\mu\text{g. P} \times 22.4/31}{\text{mg. N} \times 6} \times \frac{60}{5} = \frac{\mu\text{g. P}}{\text{mg. N}} \times 1.4$$

It would be better still not to relate the activity to the unit of weight, but to express it per mole of enzymes. This value, which can be designated as "molar enzymic activity" and for which the symbol  $\varphi$  is proposed, is:

$$\varphi = SE^{-1}t^{-1}$$

where  $S$  represents moles of substrate transformed,  $E$  represents moles of enzyme, and  $t$  is time. This is the most universal mode of expressing enzymic activity once the molecular weight of a given enzyme is known. When  $t$  is expressed in minutes, the value of  $\varphi'$  is analogous to the "turnover number" (*Wechselzahl*) introduced by Warburg for cases in which definite changes of state of the enzyme (e. g., oxidized to reduced) take place.

A tentative comparison of  $\varphi$  values for ATPase and several of the well-known enzymes has been given (19), but higher figures for the ATPase activity have since been reported. The highest value is that given by Szent-Györgyi (66), who states that one mole (arbitrarily taken as equal to 100,000) of crystallized myosin will split ten moles of ATP per second. We

thus obtain  $\varphi' = 600$ . This value is of the same order of magnitude as that of carboxylase ( $\varphi' = 850$ ) or pepsin ( $\varphi' = 1000$ ), and about one order lower than that of diaphorase ( $\varphi' = 7000$ ) or aldolase ( $\varphi' = 6600$  to  $8800$ ) but if, for the molecular weight of myosin, the figure of one to four million (73, 75) is set in the calculation, the  $\varphi$  value becomes one order higher and would be comparable to those of most enzymes involved in metabolism. Considering the uncertainty as to what is to be regarded as the molecular weight of myosin in its reaction with ATP, it is felt that the expression of the activity of ATPase in terms of  $Q_P$  is preferable for the present because it is more definite.

Bailey (1) has made the important observation that the activity of ATPase is increased several times (up to almost fivefold) when glycine buffer is used instead of bicarbonate. Alanine, leucine, serine, glutamic and aspartic acids, and cysteine gave a similar effect; sarcosine was found slightly less active. A possible explanation is that the amino acids, by virtue of coordinative-complex formation, remove traces of heavy metals, especially copper, which as already stated greatly inhibits the activity of ATPase. The highest activity values obtained under these favorable conditions were of the order of  $Q_P = 6000$ . Recomputation of Szent-Györgyi's data for crystalline myosin gives a value for  $Q_P$  of about 8000, the highest of all values reported. Because amino acid buffers were not used in his experiments, the high activity was probably due to the special care taken by the author to avoid the presence of copper in all the solutions used.

It can be said that, on the average, with rabbit myosin and bicarbonate, borate, or veronal buffer,  $Q_P$  values of about 1000 to 2000 are obtained; in glycine buffer they are three to four times higher. We owe to Bailey (1) the first comparative data of the ATPase activity of myosin from different kinds of muscle and from representatives of various species (see Table III).

TABLE III

ACTIVITY OF VARIOUS MYOSIN PREPARATIONS (1) IN CARBONATE-BICARBONATE BUFFER AT pH 9.1

Source of myosin	Number of times precipitated	$Q_P$
Rabbit (skeletal)	3	940-1050
Rabbit (cardiac)	3	230
Horse	3	720-910
Pig (cardiac)	3	170
Chicken	3	830
Toad	2	170
Frog	2	210

It will be seen that the ATPase activity of myosin from amphibian skeletal and mammalian cardiac muscle is about five times smaller than that of skeletal muscles of mammals and fowl.

The ATPase activity of most tissues has been reported by DuBois and Potter (16) not to differ very much from that of muscle. Kidney and lung gave figures only slightly lower than cardiac and skeletal muscle. Brain and smooth muscle had only about one-third of that activity. Pancreas, liver, and spleen occupied an intermediate place. Much significance cannot be attributed to these values as the determinations have been carried out on whole tissue in which different enzymes may simultaneously have been active.

### V. Identity of ATPase and Myosin

In the preceding pages it has been tacitly taken for granted that ATPase activity is a property of myosin itself. To what extent is an identification of myosin and ATPase justified? The majority of authors seem to accept the identity of ATPase and myosin, but the question cannot be regarded as definitely settled. Considering its fundamental importance, it is appropriate to summarize the arguments in favor of and against the assumed identity.

**Attempts at Preparative Separation.**—In crude myosin preparations, several enzymic activities are present apart from that of ATPase: aldolase, phosphophorases, ADPase (perhaps simulated by the presence of myokinase), etc. All these enzymes are readily removed by repeated reprecipitations, and as a rule the third precipitate is found to be practically free of all the enzymes mentioned, while the ATPase activity remains unchanged.

Ferdman (25) has found the activity of Schmidt's adenylic acid desaminase to be present in myosin preparations, and not to be separated on repeated precipitations by dilution or dialysis. Banga has reported similar observations (5), but states that the desaminase activity which accompanies myosin precipitates obtained by dilution is easily separated by salting out with ammonium sulfate.

Various methods of protein fractionation can be used: dilution with distilled water, dialysis, changing of *pH* to the acid side, and salting out. These methods can be applied repeatedly, separately, or in different combinations, and the result is always the same: the ATPase activity is invariably found in the protein which constitutes the bulk of the preparation and no separation into fractions of higher and lower enzymic activity has been achieved.

**Crystallization.**—The crystallization of protein substances cannot at

present be regarded as guaranteeing the homogeneity of the product so obtained. Nevertheless, the great value of the method is unquestionable. The fact that the ATPase activity is found quantitatively in the crystalline preparations of myosin obtained by Szent-Györgyi and rather increases on repeated recrystallizations strongly supports the view that the enzymic activity is a property of myosin itself.

**Solubility (Phase Rule) Test.**—The solubility test, based on the phase rule, is one of the most reliable criteria of purity of protein preparations, enzymes in particular (51, 53). Myosin, in this test, behaves in a peculiar manner which makes the application of the phase rule impossible. It is well known that the solubility of myosin shows a characteristic dependence on the salt concentration (60). When increasing quantities of myosin, in the form of a concentrated gel suspension, are introduced into a potassium chloride solution of a given strength (within definite limits, depending on the pH), it is found that a constant fraction of the total protein introduced is dissolved in all the samples; even with the smallest quantity of protein taken, no complete dissolving occurs, and even with the largest quantity no saturation is obtained. This is illustrated by the data of Table IV.

TABLE IV  
SOLUBILITY OF MYOSIN (22)

(Increasing amounts of a myosin gel suspension added to a constant volume of potassium chloride solution)

Expt.	0.2 M KCl				0.25 M KCl				0.3 M KCl			
	Myosin gel suspension added, ml.											
	0.05	0.1	0.3	0.5	0.05	0.1	0.3	0.5	0.05	0.1	0.3	0.5
	Per cent protein dissolved											
I	14	15	14	11.6	..	..	..	..	23.5	22	23	23.7
	13.6 (average)								23 (average)			
II	16	12.4	11.4	12.8	12.3	17.2	17.2	18	28	29.5	26	27.5
	13.1 (average)				18.4 (average)				27.75 (average)			

Thus the phase rule test cannot be carried out in its usual form, and no evidence of the presence of separate proteins of different solubility is obtained in these experiments.

The homogeneity of the myosin preparations in respect to their ATPase activity can be investigated in another way, making use of the fact that salt solutions of different strength dissolve different quantities of myosin from a given amount of the protein gel. As can be seen from Table V, ATPase activity and myosin protein behave in an identical manner, so that

TABLE V

ENZYMIC ACTIVITY OF PROTEIN IN SOLUTION AT DIFFERENT CONCENTRATIONS  
OF POTASSIUM CHLORIDE (22)

(Constant amount of myosin gel treated with constant amounts of different potassium chloride solutions)

KCl molarity.....	0.15	0.20	0.25	0.30	0.50
Protein dissolved, per cent of total..	11.6	33	50	72	100
ATPase activity of dissolved protein, per mg. ( <i>Qr</i> ).....	2450	2850	3000	2800	2600

the enzymic activity per milligram of protein remains constant, within the limits of experimental error, under the most widely differing conditions of solubility. Obviously, this strongly supports the view that ATPase is not present as an admixture, but is myosin itself.

**Sedimentation in the Ultracentrifuge.**—The data reported up to the present are conflicting. In an early study, in 1930, Svedberg found myosin preparations to be heterodisperse. At that time the extreme lability of myosin had not yet been sufficiently recognized and could have influenced the observations. Schramm and Weber (59) have reported the presence in myosin preparations of as much as four distinct fractions with different sedimentation constants.

We lack information about the relative quantities of these fractions and the distribution of ATPase activity among them, so no definite conclusions can be drawn from these observations. Moreover, the recent findings of Ziff (75) are exactly opposite to those reported by Schramm and Weber. Ultracentrifuge analysis showed myosin to be monodisperse, the sedimentation patterns giving no indication of the presence of fractions of different particle dimensions. These results are further considerably substantiated by data obtained from electrophoretic measurements.

**Electrophoresis.**—A high degree of homogeneity of myosin preparations is evident from electrophoretic observations, as reported by Bailey (1) and Ziff (75). At different pH values, in the neighborhood of the isoelectric point of myosin (6.2), as well as on the alkaline side (7.4 and 8.6), in experiments of moderate duration (6 hrs.), the electrophoresis pattern exhibited no signs of nonhomogeneity; only when electrophoresis was extended over longer periods (16 to 67 hrs.) did a small slower moving fraction appear, constituting about 15% of the total protein.

Of outstanding importance for the present discussion are the results of direct determinations of the distribution of the ATPase activity in the different parts of the electrophoresis cell and between the two fractions of dif-

ferent mobility. In a 16-hr. experiment, the activity was found perfectly uniform throughout the distance between the boundaries. The smaller, slower moving fraction, when separated after 67 hrs. of electrophoresis with compensation, was found to contain less than 9% of the total enzymic activity. Although the determination of activity values per milligram of nitrogen were not very reliable in the slow fraction because of low protein content, it is still noteworthy that the value for the main fraction, which constituted more than 90% of the protein of the preparation, was found to be almost twice as high as that of the other fraction. A significant conclusion may be drawn from these experiments: at least 90% of the total ATPase activity is found in the protein which constitutes the main part (over 85%) of myosin preparations.

Doubt could be thrown on the results of all the analytical and preparative experiments which provide evidence of the identity of myosin and ATPase by the assumption that the enzyme merely accompanies the myosin, being in some peculiar way closely associated with this protein. This objection could hardly be advanced against evidence of quite another kind which also points in the same direction.

**Thermolability.**—It has already been pointed out that the thermolability of the ATPase is strikingly similar to that of myosin. If ATPase and myosin are regarded as separate entities, then it must be assumed either that both possess the same extreme sensitivity to heat, to an extent rarely encountered among proteins and enzymes, or else that they are so closely associated that the thermal denaturation of myosin induces an inactivation of ATPase. Both assumptions are evidently rather strained.

**Action of ATP on Myosin.**—As will be discussed in more detail in the next section, the interrelations between ATP and myosin exhibit a remarkable reciprocity: myosin acts on ATP, changing it chemically, and ATP in its turn acts on myosin, changing its physical properties, such as elasticity, viscosity, and flow birefringence. It is evident that, in order to produce these changes; ATP must combine with myosin, just as it must combine, when regarded as substrate, with the bearer of the ATPase activity which is to be hydrolyzed. It seems highly improbable that two different substances are present in myosin preparations, both possessing a specific affinity for ATP; but it would be quite natural to assume that in both cases we are dealing with one and the same affinity, that between enzyme and substrate. The fact that ATP exerts a specific action on myosin is surely one of the strongest arguments in favor of the identification of ATPase and myosin.

**Artificial Admixture of ATP-Attacking Enzyme and Myosin.**—The only



attempt at an experimental demonstration that ATPase might merely be associated with myosin, but not identical with it, is represented by the experiments of Kalckar (32). Apyrase from potato was added to myosin, and, on precipitation of the protein, was found to be present in the myosin sediment to a considerable extent.

These results must be regarded as a distinct warning against rash conclusions concerning the identity of ATPase and myosin. But they are obviously very indirect in nature and cannot be taken as positive evidence which disproves the enzymic properties of myosin. As has been mentioned, precipitated once, myosin contains a number of different enzymes which are completely removed on subsequent precipitations. If all other enzymic activities are so easily eliminated, and the ATPase activity remains quantitatively in the myosin fraction, there is every reason to regard this activity not as merely associated with but as an integral property of what is at present designated by the term "myosin." If some day a separation of myosin into fractions is achieved, one possessing the ATPase activity, the others devoid of it, it would be, perhaps, a matter of academic discussion to decide which of these fractions should deserve to keep the name of myosin.

The preparations of myosin obtained at present by rather primitive and poorly specific methods of precipitation can hardly be expected to contain a single, pure substance. We obviously have to consider the probability of a certain amount of other proteins being present even in the best preparations. The results of the electrophoretic experiments show clearly that the quantity of these admixtures can be but very small. On the other hand, it is equally certain that the greatly predominating portion of such preparations is represented by an individual protein which imposes its properties upon the whole preparation. The question is whether the properties of ATPase should be ascribed to this predominant protein or to some of the minor, accompanying components. Up to the present, nothing whatever indicates that the latter should be the case. Unless direct experimental evidence to the contrary is obtained, we have the right to postulate the identity of ATPase with myosin.

## VI. Mechanochemistry

The fact that myosin, the contractile substance of muscle, possesses the enzymic properties of ATPase is significant in itself. But this fact has proved to be of still greater importance because it has served as the starting point for research on what may be designated as the "mechanochemistry"

of muscle. This term is taken to mean the study of physical changes of the contractile substance which are obviously related to its mechanical properties and are induced by chemical agents, products of the functional metabolism of muscle tissue.

The main theme of such research is clear. The newly found enzymic action of myosin cannot occur unless a combination of ATP with myosin takes place, to form a complex of the usual enzyme-substrate type. ATP is the bearer of the chemical energy generated in the course of metabolic processes and accumulated in the form of energy-rich phosphate bonds (36). Myosin is the principal bearer of mechanical properties of the muscle fiber. The fact that an interaction of these two substances has been shown to occur suggests that this very interaction is responsible also for the physical changes of myosin which finally manifest themselves in the form of muscular work. Experimental evidence has shown conclusively that these expectations, derived from the results of enzymic studies, are correct.

### 1. *Myosin Threads*

When a concentrated myosin sol is injected into pure water, it forms a thread (71). The threads can be kept several days under water in the ice box without marked alteration of their properties. Although the protein content of such threads is very low, about 2%, they possess considerable strength. Their tensile strength can easily be measured by a simple device consisting of a torsion balance with a mirror and a small clamp attached to the lever. When a load is applied by moving the torsion balance handle, the thread extends, and the extension is recorded by the displacement of the spot of light reflected from the mirror to a scale. If the degree of extension is not great (loads of about 200 mg. have usually been applied), the extension is reversible, and after the removal of the load the thread returns almost to its initial length. The action of different substances on the elasticity of the thread can be studied by immersing the thread during the test in the appropriate solution (23).

Tested in this way, ATP is found to give a specific and very pronounced effect: in concentrations similar to those present in muscle (about 0.005 *M*), ATP produces a considerable increase of the extensibility of the thread, so that under the same load the extension is 150–200% of that obtained in water or dilute saline.

Of all the substances tested, only ATP gave so great an effect in such small concentrations. However, it seems that there is a certain action common to substances possessing the phosphate anhydride structure: a substantially smaller and inconsistent, but still appreciable effect is ob-

served with ADP, as well as with cozymase (a preparation of about 60% purity), and with thiamin pyrophosphate. Inorganic pyrophosphate and

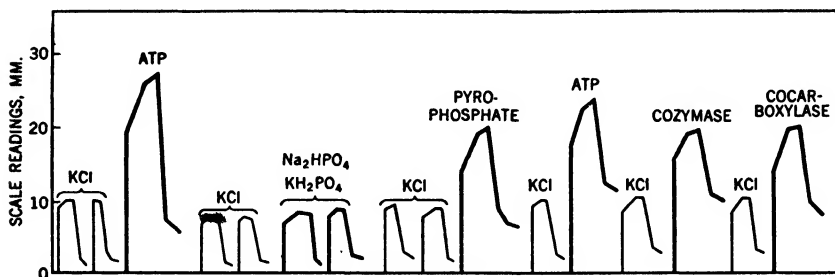


Fig. 4.—Extensibility of myosin threads (22).

metaphosphate profoundly alter the thread: the extension increases but soon becomes irreversible, and the thread breaks under loads many times smaller than those needed to break it when untreated. Inosine triphosphoric acid and inorganic triphosphate have not been tested.

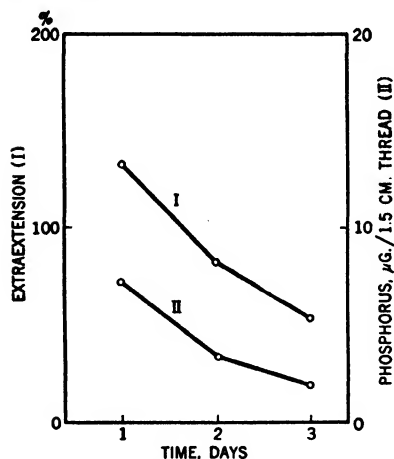


Fig. 5.—Enzymic activity and mechanochemical response of myosin threads (22). Threads kept in ice box. Curve I, decrease in extraextension effect of ATP; II, decrease of enzymic activity.

A correlation seems to exist between the enzymic activity (the latter is easily detectable in the myosin thread) and the capacity of the thread to alter its tensile strength under the influence of ATP (its mechanochemical reactivity). So, on prolonged standing, the enzymic activity of the thread is found to decrease gradually and a roughly corresponding decrease of the mechanochemical reactivity is also observed (see Fig. 5).

The same result is produced by the action of silver, which, as already mentioned, strongly inhibits the ATPase activity of myosin. It is found that silver salts abolish the reactivity of the thread; the extensibility, as such, remains practically unaltered, but is no longer influenced by ATP (see Fig. 6).

It must be pointed out that no actual contraction is observed when a thread, after being distended in ATP, is transferred, under the same load, to water or dilute potassium chloride, although, when allowed to relax with the load removed, the thread shows, on subsequent testing in these media, a return to the initial extensibility, *i. e.*, there is an increase in the elasticity

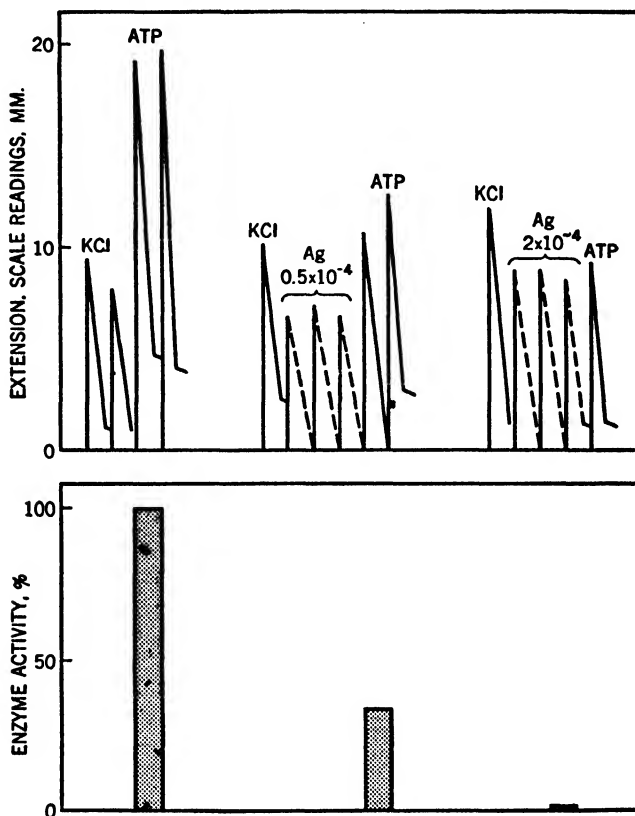


Fig. 6.—Action of silver on mechanochemical response and enzymic activity of myosin threads (22).

modulus. This peculiar behavior could be explained by the assumption that the changes in elasticity are not sufficient to overcome the resistance set up by the rather high hysteresis of the thread, and only after "recovery" in the unloaded state does the increase in tensile strength become visible.

It has been found by Szent-Györgyi (67), that the action of ATP can be seen on free, unloaded pieces of myosin threads: when placed in a dilute

solution of ATP containing definite amounts of salts (potassium chloride, magnesium chloride), the thread shrinks. In the absence of ATP, no shrinking of myosin occurs in the same solution. The effect is most pronounced on threads obtained from myosin preparations which contain a sufficient amount of actin, *i. e.*, preparations rich in actomyosin. Threads of actomyosin may shrink by 66% of their initial dimensions.

It is remarkable that the effect here is of opposite character to that which could be expected from the extension experiments, but it must be taken into consideration that the conditions of the two experiments are quite different—in one case, the resistance against an external force being measured, and in the other a free shrinking being observed. It may well be that the force producing the shrinking is negligible when compared with the stress applied by the load. What is important is that in both cases a strong and specific action of ATP on myosin is established.

The effect of ATP in producing an increase of extensibility in experiments with myosin threads could be considered as indicating that, in muscle, proteidization of ATP with myosin is involved in the relaxation phase rather than in contraction. But in the present state of our knowledge any such conclusions appear too far fetched, and therefore of little value. Much more significant are the results of observations on living muscle reported by Sandow (58) with a refined recording technique. It has been found that in the very first moment after excitation a decrease of the elastic modulus of the muscle occurs which precedes contraction. This strikingly resembles the changes of elasticity of the myosin thread under the influence of ATP. The assumption is justified that the initial, transient relaxation discovered by Sandow is the result of the primary reaction of proteidization of ATP with myosin, the further changes which ensue leading to the actual contraction.

## 2. *Viscosity and Flow Birefringence*

The observations on myosin threads have, until now, been of rather qualitative nature. Their importance consisted in the demonstration, for the first time, of the action of ATP on the physical properties of the contractile substance of the muscle. Other manifestations of this action are revealed by the study of the influence of ATP on the characteristic properties of myosin solutions. Here much more precise quantitative methods have been applied. Valuable information in this respect has been brought forward by the work conducted independently and almost simultaneously in Cambridge and in Szeged.

The results obtained by both groups of investigators are qualitatively in complete accord. It is found that the two most characteristic properties of a myosin solution, namely, high viscosity and flow birefringence, are profoundly influenced when ATP is added to such a solution.

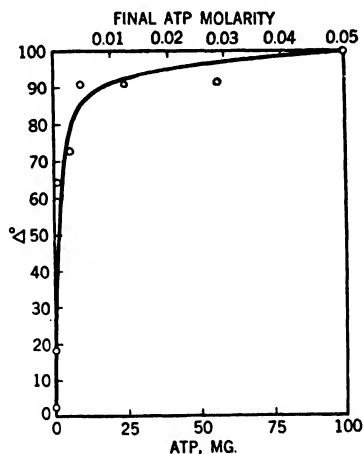


Fig. 7.—Changes in flow birefringence of myosin solution as a function of ATP concentration (49). All readings at 560 r.p.m. and 20° C.

ATP, mg.	Fall of $\Delta^\circ$	Per cent of maximal
100	55	100
50	50	91
25	50	91
10	50	91
5	40	73
1	35	64
0.1	10	18
0.01	1	2

The experiments of Needham and co-workers (12, 49), performed with an elaborate technique of high precision, are particularly instructive. Of the two properties of myosin which depend on the shape and dimensions of the protein particles, flow birefringence shows the greater change. An average decrease of about 50% is observed with concentrations of ATP comparable to those present in muscle. The dependence of the effect on the concentration of ATP is shown in Figure 7. An almost linear increase of the decrease in birefringence is observed up to a concentration of about 0.005 *M*, where the maximum effect is practically attained; the curve here shows a sharp break so that the fall of birefringence is not further materially changed even by a tenfold increase of the concentration of ATP.

The viscosity has been found by the British authors to be influenced in a less drastic way. The anomalous character remains unchanged, but the relative viscosity decreases by some 15%. Considerably larger changes of viscosity are reported in the papers of the Szeged group, evidently because preparations of much higher actomyosin content have been used, and according to Szent-Györgyi, it is precisely the actomyosin that is responsible for the whole effect (3, 61).

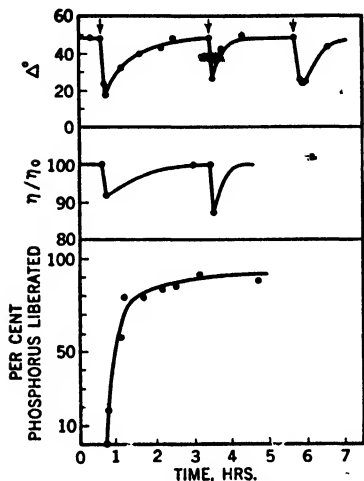


Fig. 8.—Changes in flow birefringence, relative viscosity, and liberation of phosphorus observed on addition of ATP to a solution of myosin. Arrows indicate addition of 50 mg. ATP; viscosity changes are given in per cent of initial viscosity.

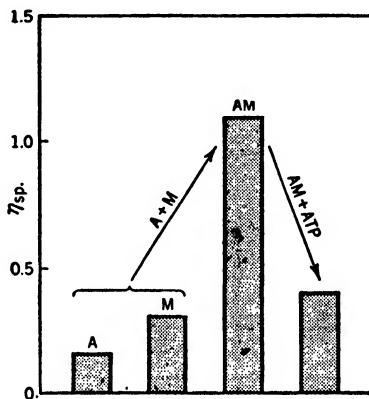


Fig. 9.—Effect of ATP on viscosity of actomyosin: 0.7 mg. per ml. actin (A); 1.75 mg. per ml. myosin (M); and 0.015% ATP.

The effects produced by the addition of ATP gradually decrease with time as the ATP is split by myosin; further addition of ATP makes the whole phenomenon reappear. In Figure 8, taken from the paper of Needham and co-workers (12), an experiment is represented in which changes of flow birefringence and of viscosity, as well as the splitting of ATP, were recorded simultaneously. It gives a remarkable picture of the characteristic, fully reversible effect of ATP.

The decrease in viscosity produced by ATP was found by Szent-Györgyi (66) to depend mainly on the amount of actomyosin present in the preparation used for the experiment. The usual preparations of myosin em-

played by all previous workers were very poor in actomyosin, for only small amounts of the latter are liberated from the muscle tissue during a short extraction. If the duration of extraction is extended over twenty-four hours, preparations are obtained (originally designated as "myosin B") which are very rich in actomyosin. The effect of ATP is very great on such preparations, or on actomyosin solutions, obtained by mixing pure myosin and actin. This effect is regarded by Szent-Györgyi as due to a dissociation brought about by ATP of actomyosin into its components. Figure 9, redrawn from the data of Szent-Györgyi (64), shows both the great increase in viscosity obtained on mixing myosin and actin, and the great decrease in viscosity, back to the level of the sum of the viscosities of the components produced by ATP.

Recent experiments by Rubinshtein and Petrova (56), made on preparations rich in actomyosin ("myosin B"), confirm the finding of Needham *et al.* that the anomaly of viscosity is not influenced by ATP, even in cases in which the changes in relative viscosity are very great.

The phenomena of viscosity and of flow birefringence are both related to purely mechanical forces. Accordingly, the changes produced by the chemical agent, ATP, may be designated as mechanochemical effects. These effects, observed on myosin solutions, are even more specific than the changes of elasticity found on myosin threads (12, 49). Several substances which influence the tensile strength of the thread, although to a smaller degree, or in another way than ATP (ADP, thiamin pyrophosphate, inorganic pyro- and metaphosphates) were found to have no effect on flow birefringence; nor have any other metabolites been found active, such as lactate, hexose phosphates, phosphoric esters of glycerin and glyceric acid, phosphocreatine, adenylic and inosinic acids, diphenyl phosphate, and hexametaphosphate. Only inosine triphosphate, which, as already pointed out, is split by myosin even more easily than ATP, affects the physical properties of the protein in the same way as does ATP. Noteworthy is the behavior of inorganic triphosphate. As will be remembered, this compound seems also to be split enzymically by myosin. The salt as such has no effect on the birefringence, and, moreover, inhibits to a considerable degree the effect produced by ATP. This can be interpreted as a result of competitive interaction: evidently both substances possess an affinity for the same groups in the myosin molecule, but only ATP produces the characteristic changes of the physical properties of the protein, the presence of the purine-ribose structure being essential for this effect.

Thus it can be said that the mechanochemical effect ultimately depends on the specific enzyme-substrate affinity between myosin and ATP, and



the admirable definition of myosin as a "contractile enzyme" given by Needham (49) appears to fit the facts perfectly. This view would gain further substantial support if it could be shown that changes of the enzymic activity of myosin are accompanied by corresponding changes of the mechanochemical effects observed on myosin solutions, in the same way as this has been established on myosin threads. But such experiments have not yet been reported.

### 3. *Stoichiometry*

The discovery of an interaction of ATP with myosin which was detectable by physical methods seems to offer the opportunity of determining the quantitative relations between the two reacting substances. However, the discrepancies between data reported by different workers are so great that it is not possible to arrive at any definite conclusions. According to Mommaerts (44), a maximal effect on viscosity and on flow birefringence (and in a less definite way on the volume of a myosin gel also) is obtained with about 0.5 mg. of ATP per 100 mg. of myosin. Thus, one mole of ATP would react roughly with 100,000 g. of myosin; this figure is regarded as the molecular (more correctly the combining) weight of myosin and it is taken that one mole ATP reacts with one mole of myosin.

Corrected for admixtures present in the preparations of Mommaerts, the figure of 70,000 is now given by Szent-Györgyi for the molecular weight of myosin (66).

On the other hand Needham *et al.* (12, 49) arrive at a different figure—of the order of 30 moles of ATP per 67,000 g. of myosin. More detailed investigations with systematic variations of quantity and concentration of both reactants are needed before an explanation of these discrepancies can be given; indications from the enzymic side, in the form of determinations of the Michaelis constant, might also prove useful.

### 4. *Myosin Monolayers*

The wonderful properties of muscle are determined not only by the chemical properties of the substances involved in its function, but also, and perhaps in no smaller degree, by the physical structure in which these very substances are arranged in the muscle fiber. A solution of myosin is quite appropriate for the study of the chemical behavior of the contractile protein, but a wide gap separates the properties of a myosin solution from those of the contractile element of muscle tissue. Great difficulties arise as soon as we try to obtain a picture of the work of a muscle fibril by proceeding from observations obtained on myosin solutions. The difficulties are at least the same as if one were to draw conclusions about the mechanical

properties of a strip of paper by studying the properties of a suspension of paper pulp in water. It cannot be denied that much valuable information can be obtained from such a study, but the vagueness of the final deductions is obvious.

It might seem that the myosin thread represents a much more adequate model for mechanochemical studies. This is not quite so. Two circumstances contribute to make the myosin thread but a very remote and imperfect model of the muscle fibril. The myosin has in the thread very little, if any, orientation; moreover, the protein particles are extremely loosely packed, being divided by the enormous amount of water of hydration; as already mentioned, the myosin content of a thread is not more than roughly 2%, with 98% water. In the fibril, at least six to ten times less water is enclosed in the protein structure, which is of a highly ordered nature. Close packing and orientation are the two most important features which an adequate model of the muscle fibril must possess. It seems that the only system that would satisfy these requirements is a monolayer. This is one of the reasons why the study of myosin monolayers appears to have important advantages over the study of myosin solutions or even myosin threads. The state of myosin organized in the two-dimensional space of the monolayer would be the closest approximation to the three-dimensional organization existing in the muscle fibril.

The objection that surface spreading is accompanied by a denaturation of the protein, due to uncoiling of the corpuscular molecule, is not very serious. Evidence is accumulating that many enzymes do not lose their specific activity when spread as monolayers, and the view has also been advanced that proteins in the native state possess a sheetlike structure (14).

It appears that only one attempt to study the properties of myosin monolayers as influenced by metabolic factors has as yet been reported. In 1935, Rideal and co-workers (46) published a brief report on the measurement of area and surface potential of myosin monolayers. Changes produced by lactate were recorded. The results obtained evidently did not bring much satisfaction, and work in this direction seems to have been discontinued. We cannot attribute to lactic acid the role it occupied as "active substance" in the theories of muscular contraction at the time of the experiments of Rideal. But the idea on which these experiments were based is fundamentally sound and fruitful. They may be regarded as the very first case of a mechanochemical investigation, and ought to be developed further. A study of the mechanical properties of the monolayer, rather than measurements of area, seems to be particularly desirable.

According to Gorter (27), myosin can be spread only after being treated

with trypsin. Such material would be of little value for the study of any properties of the protein. But it has been shown by Dervichian (13) that myosin can be readily spread when applied in a solution containing a small amount of some volatile or soluble surface-active substance, *e. g.*, 0.1% of amyl alcohol. The layers so obtained possess a high surface viscosity (see Fig. 10). Since work in this direction is still in progress, no new statements about the character of interaction between ATP and myosin can as yet be made. That this line of approach is promising can be inferred

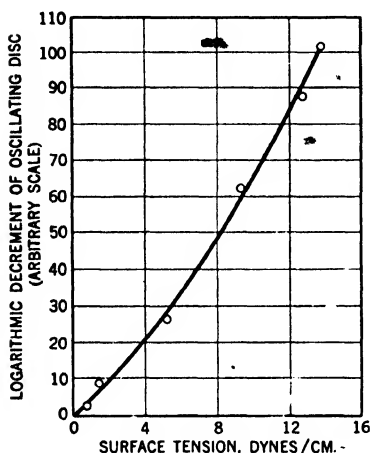


Fig. 10.—Surface viscosity of myosin monolayer as a function of surface tension (0.025 mg. myosin).

from the observations made by Harkins (29) on an enzyme of quite different character, catalase. When the enzyme is spread as a surface layer, it is found that an interaction of the enzyme with the corresponding substrate, hydrogen peroxide, is accompanied by substantial changes of the layer, its thickness being decreased by 4 to 9 Å.

## VII. Role of ATPase in Cells Other Than Muscle

The view is advanced by most of the research workers (*cf.* 36) that the role of ATP as the ultimate bearer of chemical energy utilized for the physiological functions of the cell is by no means restricted to the muscle tissue only. *But if we look for experimental evidence in favor of this statement, we find it almost completely lacking.* The same can be said of the enzyme, ATPase, whose task it would be to liberate the energy of ATP in such a way as to make it available for the performance of certain cellular functions. In a recent review, Potter (54) summarizes the present state of affairs by saying that it is not possible at present to discuss the manner of function of ATPase in tissues other than muscle. Perhaps the situation is not as hopeless as that. It appears that, in a few cases at least, some evidence is seen of the participation of ATPase in definite cellular functions.

### 1. Spermatozoa

One such case is that of spermatozoa. It was found almost simultaneously by Lardy, Hansen, and Phillips (35) and by Burnascheva (10, 20)

that ATP plays an equally important role in the motility of these cells as in the contraction of muscle. ATP is present in the sperm cell in a considerable amount as compared with that of muscle. Under normal conditions, the level of ATP is maintained constant by a continuous synthesis which occurs at the expense of oxidation of phospholipides (35) or glucose breakdown. When resynthesis is stopped (cyanide poisoning in glucose-free medium) the amount of ATP rapidly decreases and, as soon as the supply initially present is exhausted, the cells are found to become motionless. On addition of glucose, ATP is resynthesized and motility reappears. The cells can be immobilized even when ATP is still available, if its enzymic breakdown is inhibited by magnesium salts (magnesium narcosis of the cells).

The splitting of ATP is of the apyrase type; it cannot yet be decided whether the effect is due to the function of one single enzyme, or to a combined action of several enzymes. From a homogenate of sperm cells a protein fraction ("spermosin") has been isolated, possessing enzymic activity toward ATP (20). But only a part of the total activity of the homogenate is recovered in this protein fraction, and further progress is to be awaited before any conclusions can be made about the possible role of "spermosin" as the contractile substance of the spermatozoan tail. Nevertheless, the importance of the enzymic splitting of ATP for the motility of the sperm cell may be regarded as securely established.

## 2. Retina

Observations have been reported from time to time which indicate that the metabolism of phosphorus compounds is in some way involved in the visual function (34, 45, 55). The retina has not been among the tissues studied by DuBois and Potter (16) for ATPase content. The ATPase activity of the retina was determined by Venkstern (70) and found to be appreciable, though not very high. When the tissue is treated in the manner usually employed for the extraction of rhodopsin—*i. e.*, tanned with alum, washed, and extracted with detergents such as bile salts, saponin or digitonin—the ATPase activity is found to go over almost quantitatively into the rhodopsin solution. It is tempting to regard this association, not as an occasional one, but as indicating some participation of ATPase in the primary processes of the photochemical phase of the visual act, when radiant energy generates the chemical agents which produce the nervous impulse. This view would find substantial support if any definite differences could be demonstrated between dark- and light-adapted retinas, but it

must be stated that so far no consistent results of this kind have been obtained.

### 3. Yeast

The role of ATPase in the normal metabolism of the yeast cell has recently been made evident by Meyerhof (43). It was suggested some time ago, but without experimental proof, that the Harden-Young equation for cell-free fermentation is due to the absence of ATPase which must be present in the undamaged yeast cell (18, 42).

One of the characteristic features of the Harden-Young type of fermentation is the very slow rate at which hexose diphosphate is fermented by cell-free preparations, when no glucose is present to serve as phosphate acceptor from ATP. It has been presumed that, in the living cell, the "depolarization" of the adenylic system, *i. e.*, the regeneration of the less phosphorylated members which can serve as phosphate acceptors, is effected by the action of ATP-dephosphorylating enzymes. The absence of the latter in ordinary cell-free preparations would be responsible for the Harden-Young equation. The correctness of this view has been demonstrated by Meyerhof in an elegant manner. It was shown that the addition of a powerful adenylypyrophosphatase from potato brings the rate of hexose diphosphate fermentation to the maximal level, otherwise obtained only with free sugar or in presence of arsenate. A high ATPase (or apyrase) activity was also demonstrated in the cellular mass of yeast preparations.

Thus the Harden-Young equation, which had governed cell-free fermentation since the discovery of zymase, has been changed back, by the mere addition of ATPase, to the Gay-Lussac equation typical for fermentation by living yeast cells. Obviously this function of ATPase as an "anti-Harden-Young factor" must be regarded as of widest occurrence. It will come into play in all those cases in which ATP acts primarily as a "mobile coenzyme" (52) of carbohydrate breakdown, and not as a carrier of energy for the performance of some special functions of the cell.

### VIII. Conclusions

The quantity of myosin in muscle is so high, and its enzymic activity so considerable, that the question arises as to the way in which a coexistence of ATP and myosin becomes possible.

Taking a value of  $Q_p = 6000$  for the ATPase activity (the highest found by Bailey, but lower than that reported by Szent-Györgyi), and a content of 100 mg. of myosin per g. of muscle, we find that 1 g. of muscle would liberate about 14 mg. of phosphorus from ATP per minute. Considering

that only one phosphate residue of ATP is involved, this would correspond roughly to one hundred times the amount of ATP present in 1 g. of muscle, which is of the order of 0.3 mg. 7'-P. In other words, all the ATP contained in muscle could be split by the myosin in less than one second.

A rough estimate of the actual rate of ATP-splitting in resting muscle can be obtained from the increase in orthophosphate after iodoacetate poisoning and under anaerobic conditions, when the breakdown of ATP is no longer overshadowed by any resynthesis. The values found are of the order of 30 to 50  $\mu$ g. of phosphorus per gram of muscle per minute, *i. e.*, several hundred times slower than could be expected from the potential myosin activity.

Obviously, in muscle, some conditions exist which greatly reduce the interaction between myosin and ATP, at least during rest. One explanation which has been advanced (1) is that myosin in resting muscle is enzymically inactive, due to a local absence of calcium, known to be necessary for the ATPase function of the protein. Thus, even if ATP were bound to myosin in the resting state, no splitting would occur until calcium is released from its bound form and comes into contact with the myosin-ATP complex.

It is only natural to ascribe to calcium activation an important role in muscular dynamics, but it seems probable that other mechanisms are of greater importance. One of them would be the spatial separation of ATP from myosin during rest, as postulated by Caspersson and Thorell (11) who found that the adenylic compounds in resting muscle are localized in the isotropic disc and migrate into the anisotropic segment during activity. These shifts are undoubtedly of great significance, but the assumption that myosin is localized only in the anisotropic disc appears questionable. The current view is that both morphologically distinct parts of the muscle fibril are built up of myosin, the optical differences being due to the state and orientation of the protein micelles rather than to differences in the distribution of myosin.

Still other conditions must be responsible for the inaccessibility of ATP to the action of myosin. Of these the conjugation to proteins other than myosin seems to be the most plausible. No transport over distances—rather considerable when compared with molecular dimensions—would be necessary, as the proteins could be in close proximity, almost in immediate contact with each other. It would be a "protective conjugation," of the kind observed, for example, with thiamin pyrophosphate, where the free compound is rapidly hydrolyzed by phosphatase, but becomes completely resistant to the action of the enzyme when bound to its specific

protein partner, apocarboxylase (24). We already know at least six enzyme-proteins that will compete with myosin for an interaction with the adenylic compounds: the proteins of the phosphophosphatases involved in transphosphorylations with hexose; hexose monophosphate; 1,3-diphosphoglycerate; phosphopyruvate; creatine; and, furthermore, myokinase. The distribution of the apoteons between all these will depend primarily on the relative affinities.

If it is taken that the interaction of myosin with ATP is the fundamental phenomenon responsible for muscular activity, then the following, at present purely tentative, scheme of the successive events could be proposed. During rest, ATP is held fast by proteins other than myosin. The effect of the stimulus is one of "transproteidization": ATP is released from the proteins with which it was conjugated during rest, and is taken over by myosin. The proteidization of myosin with ATP produces the molecular or micellar changes responsible for the mechanical effects. Next, the catalytic activity of myosin comes into play; ATP is dephosphorylated; ADP, having a lower affinity for myosin, dissociates; myosin becomes deproteidized and returns to the initial physical state. ADP is taken over by other enzymic proteins, becomes rephosphorylated in the processes of recovery metabolism, and the cycle is completed. In this scheme, the essence of muscular activity is the process of transproteidization, which is governed by changes in affinity between the various proteins and the corresponding apoteons (ATP and ADP).

One of the main points of this scheme is the assumption that there is a substantial difference between the affinities of myosin for ATP and for ADP; however, no direct determinations of the corresponding dissociation constants have yet been reported; the assumption just mentioned can be based on certain analogies and indirect evidence only. The classical research of Warburg on the hydrogen-transporting enzymes has made evident the significance of changes in affinity between protein and prosthetic group, resulting from changes in degree of hydrogenation. There is every reason to expect that, in the present case, no less an influence on the protein-apoteon affinity will be exerted by changes in the phosphorylation level. This, of course, does not exclude the possibility that various other factors (*e. g.*, ionic influences) also contribute to a certain degree to such changes in affinity.

The affinity of myosin for ATP must be great, as is demonstrated by the character of the curve of enzymic splitting of ATP: it is linear with time, almost to the point of exhaustion of the substrate, the enzyme evidently being saturated even at very low concentrations of the substrate. Data of

two kinds can be used to obtain indications about the possible absolute value of the affinity of myosin for ATP. The measurements of Needham *et al.* (12) (see Fig. 7, page 179) show that half the maximal effect of ATP on the birefringence of myosin is obtained at an ATP concentration of about 0.001 molar. On the other hand, from the results reported by Mommaerts (44) it appears that half the maximal effect of ATP on the viscosity of "myosin B" is obtained at an ATP concentration of the order of 0.00003 molar. The data obtained by the two methods are so divergent that an exact value of the corresponding dissociation constant cannot be defined; but it appears that this value is not more than  $10^{-3}$ , and is perhaps as low as  $3 \times 10^{-5}$ .

No data whatever are available for the estimation of the absolute value of the affinity between myosin and ADP, but certain indications can be obtained about the *ratio* of the affinities of myosin to ATP and to ADP, respectively. Kalckar (32) has found that ADP inhibits the splitting of ATP by myosin. The inhibition is obviously a competitive one. In the presence of an equimolar quantity of ADP the splitting of ATP is inhibited by some 25%. If both substances had the same affinity to myosin, the protein would be distributed equally between them in an equimolar mixture, and the inhibition would be 50%. The observed inhibition of 25% means that the affinity of myosin for ADP is nearly four times smaller than for ATP. Consequently, when ATP is dephosphorylated by the enzymic action of myosin, the resulting ADP will readily escape from the protein, a deproteidization of myosin will occur. It can be said that proteidization of myosin is produced by its enzymic affinity and is abolished by its enzymic activity.

If the role of the immediate moving force in muscular activity is attributed to the ATPase function of myosin, it may be asked whether this enzymic activity is sufficient to provide the energy actually expended during the work of muscle. If the figure reported by Szent-Györgyi for the activity of myosin (65) is taken, namely, that one mole of myosin splits ten moles of ATP per second, it follows that the myosin in 1 g. of muscle (roughly 100 mg.) can liberate about 0.3 mg. of phosphorus from ATP per second. This corresponds to 0.12 cal. per g. muscle per sec.—a value sufficient to cover the heat production of muscle during tetanic contraction, which appears to be of the order of 0.1 cal. per g. per sec. or less.

Too much significance should not be ascribed to computations of this sort; nevertheless the result decidedly lends additional support to the view that the ATPase activity of myosin is of paramount importance for the function of muscle.



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# STATES OF ALTERED METABOLISM IN DISEASES OF MUSCLE

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## I. Introduction

Striated muscle makes up about 42% of the total weight of the body. It is affected by a variety of diseases which in most cases can be divided sharply into two categories: (1) in which the primary process appears to be located in the muscle fibers, and (2) in which the primary process is located in the central or peripheral nervous system, with resulting secondary changes in muscles innervated by the affected nerve cells. The problem of the secondary affections of muscle is concerned with the complex relationship between the integrity of innervation of muscle and the maintenance of the optimum state of nutrition of the muscle cells, and cannot be sharply divorced from a consideration of those diseases affecting the muscular system in which no neurological component is recognized.

It seems strange that so little should be known concerning the mechanism of disease in an organ such as muscle, about which more exact fundamental information regarding normal mechanisms is available than for any other organ. Certainly studies of the pathophysiology of muscle disease have not

kept pace with the phenomenal advances in the field of physiology and biochemistry of normal muscle. Nor has a study of the diseases of muscle thrown any particular light on normal processes of muscle in any way comparable, for example, to that which a study of diabetes shed on carbohydrate metabolism. It is only very recently that a study of muscle disorders has begun to receive impetus from several new and important sources of information. The discovery of a disease of dietary origin in animals, which resembles progressive muscular dystrophy but which, unlike the human disease, can be cured with vitamin E, is influencing the study of affections of muscle in man. The observation that prostigmine relieves to some extent the symptoms of myasthenia gravis has accelerated fundamental studies on the metabolism of acetylcholine and cholinesterase in this disease. The discovery of an aberration in potassium metabolism in periodic muscular paralysis has led to effective therapy for this disorder, and to basic studies on the relation of changes in concentration of potassium in the blood and tissues to disturbances of muscle function. In the future, therefore, we may expect that increasing knowledge of the physiology of muscle, particularly that concerned with the chemistry of contraction, transmission of nervous impulse to muscle, and the mode of action of specific pharmacological agents on nerve, muscle, and the myoneural junction, will provide us with new and more effective means for an attack on the whole field of the muscular disorders.

Lack of basic data, however, has not limited speculation and wild guesses concerning the nature of the mechanisms underlying the pathology of the muscle syndromes. The literature has been abundant on diseases of muscle, beginning around the start of the present century, and has been growing yearly ever since. Many excellent reviews have been published, but these have dealt mainly with the clinical and pathological aspects of muscular disorders, and only superficially, if at all, with biochemical and physiological studies. The present review, therefore, will attempt to collect and correlate, in so far as possible, such pertinent data as have been encountered in the scattered publications dealing with states of altered metabolism in the whole heterogeneous group of disorders of muscle in man and in animals.

Protoplasm in general is endowed to a variable extent with the property of contractility, but it is in muscle that we encounter this property in its highest state of development. Muscle cells are elongated structures which are capable of rapid change in form without significant alteration in volume. It is the property of changing form which enables groups of muscle cells, acting synergistically, to move parts which may be near or fairly remote from the contracting center. The cells are composed of long contractile threads, or myofibrils, which rest in a less differentiated protoplasm, the sarcoplasm. Many of the myofibrils present alternating

isotropic and anisotropic areas which give voluntary muscle its characteristic striped appearance. Other myofibrils appear to be homogeneous throughout their length. In certain myopathies, marked changes occur in the gross structure of the muscles which can be correlated roughly with the degree of loss of muscle function observed in these syndromes. In other disorders of muscle, however, there occur few, if any, histological changes, although gross changes in function are readily apparent. Since it is difficult to discuss aberrant metabolism in disease without at the same time attempting to relate the physiological alterations to structural change in tissue, brief reference will be made whenever possible to the nature of the pathological lesions in muscle which accompany the muscular disorders. Although muscle may be divided broadly into two types, striated and smooth, we shall be concerned here, in so far as states of altered metabolism are concerned, with the former, since no well-defined syndromes having their origin in smooth muscle have been described.

## II. Muscular Atrophy

It has been known for many years that both disuse and motor denervation produce atrophic and degenerative changes in skeletal muscle. Injury to a neuron may lead to extensive degeneration of the corresponding area of innervation in muscle, as may also injury to another neuron which joins the latter and activates the first by synapse (217). It is not known, however, whether the subsequent degeneration results from the diminution in activity of the second neuron, or whether there is some subtle metabolic relationship between the two which makes maintenance of the original state of the second neuron dependent on the metabolic integrity of the first.

Numerous changes occur in the chemical constituents of muscle as a result of motor denervation, disuse through section of the tendon, or immobilization of the muscle by means of casts. These changes can largely be accounted for by progressive alteration in the relative quantities of muscle cell and connective tissue phases (126). Generally these changes are limited to those constituents which show differential concentration in normal muscle. Muscle glycogen, however, has been shown to present an exception to this generalization. Upon denervation, muscle glycogen is said to undergo a decrease in concentration at a velocity far exceeding the rates of differential change in muscle phases (96). The instability of glycogen in denervated muscle is further evident in the observation that it is more susceptible to the glycogenolytic effects of exogenous adrenaline,

insulin, and thyroxine (98). In a search for possible causes of altered glycogen metabolism exhibited by muscles undergoing atrophy from denervation, comparative studies have been made by Lazere, Thomson, and Hines of changes in glycogen concentration in muscles undergoing atrophy from causes other than denervation, including tenotomy and immobilization by casts (126). Determination of the content of glycogen and creatine, the degree of fibrillation, and measurements of tension and weight of the muscles following denervation, tenotomy, and immobilization were carried out at frequent intervals on the hind limbs of rats, with intact muscle of the contralateral member serving as the control. The fall in concentration of glycogen was comparable in degree in the affected muscles regardless of the type of experimental procedure. In all instances the rate of decrease in glycogen concentration was much more rapid than the rate of diminution of creatine, contractile strength, or weight. Conversely, restoration of muscle function through regeneration of the crushed nerve, reattachment of the tendon, or removal of the cast, was associated with much greater velocity in the rate of redeposition of glycogen than recovery of muscle mass, strength, and creatine concentration. As a result of the striking similarities in the time relationships observed for the change in glycogen concentration in the three types of muscle atrophy and subsequent regeneration, the authors suggest that the factor provoking these changes may be common to the phenomena of denervation, immobilization, and tenotomy. Moreover, it is suggested that the factor resulting in the disappearance of glycogen may be a decrease in metabolism associated with reduced tension in the muscle, and that the absence of normal muscle contractions may possibly have resulted in energy levels insufficient for glycogen synthesis. The observation made by Fischer (62) that electrical stimulation delays atrophy of denervated muscle, is cited as lending weight to this hypothesis.

Few studies have been made on the respiratory metabolism of muscle undergoing atrophy as a result of denervation or disuse. Results of experiments on oxygen uptake of atrophic muscle have been reported by Langley and Itagaki (123). Hines *et al.* (100) have reported results of studies on the respiratory quotient of intact and denervated muscle. Studies have also been made on glycogenolysis, lactic acid formation, and succino-dehydrogenase activity (118). The oxygen consumption of muscle was taken as an indication of the respiratory activity. Attention was called to the fact, however, that mechanisms other than oxygen-activating enzymes may limit the rate of oxygen utilization. Among these are the hydrolytic enzymes concerned with the cleavage of compounds in muscle.

and the formation of substrates for oxidation, and systems which in the presence of suitable oxidizing substances permit the catalysis of hydrogen substrates. In the results of experiments reported by Knowlton and Hines (118), comparable rates of oxygen consumption were found for atrophic muscle and contralateral controls. The values for normal muscle were in good agreement with those reported by others (52, 183, 201). Oxygen consumption, measured by two methods, for muscles which had been undergoing atrophy for 28 days, were 108 and 114% of the normal. These results suggest that oxidative substrates occur in adequate amounts in denervated muscle after comparatively long periods of time. It is pointed out that the loss of muscle substance in atrophy is chiefly in the cytoplasm.

The oxygen consumption of fascia and other connective tissue constituents of muscle is known to be low. In atrophy, the relative increase in the connective tissue phase of muscle should call for lowered oxygen consumption. The fact that it is unchanged, or slightly increased, therefore, makes it likely that the rate of oxygen consumption of the non-connective portion of the muscle increased as the atrophy progressed. Knowlton and Hines (118) explain this relative increase as being due to an increase in the number of nuclei per unit weight of atrophic muscle, since it is known that an increase in nuclei is approximately proportional to the weight loss, and likewise to the increase in connective tissue. In the experiments of Langley and Itagaki (123), the *in vivo* oxygen consumption of muscle following denervation was found to be increased. Knowlton and Hines (118) suggest that this may have been due to the fibrillation which is shown by denervated muscles *in situ*. In isolated muscle, on the other hand, fibrillary contractions disappear very soon following the removal of the muscle from the animal, and could not, therefore, have occurred in the experiments which they describe. In these experiments it was found that the glycogen disappearing during the first thirty minutes of anaerobic glycolysis at 37° C. was the same per unit weight for normal and denervated muscle. Atrophic muscles, however, as previously cited were found consistently to have a lower glycogen content than contralateral control muscles. While confirmatory of previous results obtained by these investigators (96), the report is in contradiction to the results obtained by most other workers (11, 37, 115, 138, 219). The authors suggest that the discrepancy between their results and those of others is due possibly to the use of different species of animals, and perhaps to differences in the methods of securing muscle.

Freshly excised, denervated muscle is claimed to be consistently higher



in lactic acid content than control muscles from the same animal (118). This is attributed to fibrillary activity exhibited by freshly excised muscle. Among other changes said to occur in muscles undergoing degeneration from inactivity is an increase in glutathione content (114, 170).

Results of studies on constituents of the blood before and after passing through denervated muscles have not revealed values differing from those obtained on arterial and venous blood from intact contralateral controls (100). In these experiments, section of the sciatic nerve was made, and bleedings performed under anesthesia from the femoral artery and vein over periods of one-half hour to fourteen weeks afterward. Values for glucose, lactic acid, oxygen, and carbon dioxide content of arterial and venous blood were not significantly different from those found in the arterial and venous blood of control muscles. The authors conclude from these data that an intact nerve supply does not determine the mixture of substrates oxidized, and that the metabolism of denervated muscle is qualitatively the same, in so far as can be determined by analysis of blood constituents, as that for normal muscle. Sato and Kasugai (186), however, present results of analyses on arterial and venous blood which they interpret as showing that atrophic muscle, whether due to degeneration after nerve section, or inactivity following section of tendon, takes up more lactic acid and oxygen from the blood than muscles in which the nerve supply is intact. Moreover, the differential response of muscle with respect to uptake of lactic acid and oxygen was found to be considerably magnified when lactic acid was introduced in large amounts into the blood. The investigators further report that there is an increased rate of glycogen synthesis in the affected muscles, which is believed to account for the accelerated uptake of oxygen and lactic acid. Similar results on oxygen uptake of denervated muscle have been reported by Hirohasi (101).

Extracts from atrophic muscle are reported to show marked impairment of their capacity to effect the decolorization of methylene blue in the presence of sodium succinate (118). The phenomenon is claimed to occur early in the course of atrophy from denervation, and is believed to indicate a loss of about 45% of the content of succinodehydrogenase. This finding contrasts markedly with the fact that no impairment was found early in the development of atrophy in the consumption of oxygen, glycogenolysis, and in the formation of lactic acid. Since these latter phenomena are undoubtedly dependent fundamentally on the integrity of certain enzyme processes, we must, if we accept this observation, assume that a differential effect of denervation is exerted on the concentration of succinic dehydrogenase in muscle. An alternative explanation, not considered by the

authors, and admittedly labored, is the possibility that the changes in muscle phases which occur with atrophy may produce sufficient structural change in the muscle to result in a less efficient extraction of succinodehydrogenase.

Results of a number of studies indicate that the more active the skeletal muscle, the higher is its concentration of phospholipide (19-22). In muscular atrophy and dystrophy, which are primarily myopathic in origin, there may be marked diminution in the concentration of phospholipides (19, 39). With respect to studies on the phospholipide content of muscles after denervation, results are contradictory. No changes were found by Grund (77), while a decrease in phospholipide concentration of denervated muscle was reported by Cahn (31) when calculated as per cent of dry, defatted tissue. Artom (9), on the other hand, working with radioactive phosphate, found the lipides, newly formed phospholipides, and to a less extent, the total phospholipides, in denervated muscles to be increased. The increase was apparent in a determination on fully purified phospholipides, and was even more increased when values were calculated on the basis of dry muscle protein. Artom's results show, moreover, that phospholipides of the muscle are formed in the liver from plasma phosphate, and carried to the muscles by the plasma. In all cases, larger amounts were deposited in the denervated muscles than in intact controls. Similar results were obtained following the introduction, intravenously, of an emulsion of labeled liver phospholipides. In an attempt to explain the differences in behavior of phospholipides in muscular atrophy and dystrophy from various causes, Artom suggested that muscle alterations which are primarily dependent upon loss of nerve control may be accompanied by an increased deposition of "lipometabolic" phospholipides, and other lipides, whereas atrophy originating primarily in the muscle is perhaps followed by a decrease in "protoplasmic phospholipides." This hypothesis would limit Bloor's concept of a parallelism between the phospholipide content of skeletal muscle and its activity to examples of atrophy and dystrophy not occasioned by denervation. It would not, however, reconcile the results of studies on the phospholipide in muscles undergoing dystrophy from vitamin E deficiency. In this syndrome, which appears to be primarily muscular, the phospholipides are reported to be markedly increased (99, 160).

A number of studies have been made in an attempt to learn the effect of various conditions upon the rate of atrophy in muscles which have been subjected to denervation. Acetylcholine and hypercalcemia induced by parathyroid extract were without effect on the rate of weight loss of

denervated muscle (99). The rate of atrophy following tenotomy was somewhat slower than that due to denervation. When tenotomy was combined with denervation, the rate of atrophy was equal to that produced by denervation alone. Electrical stimulation and exercise of muscle by passive extension were not found to influence the rate of atrophy. The investigators conclude from the results of these experiments that tension and activity which is elicited artificially cannot be substituted for the neurotropic effects of intact innervation. The administration of glycine was not found to affect the rate of atrophy. Also no effect of temperature on the rate of atrophy following denervation could be elicited (97). The rate of atrophy in denervated muscles of animals kept at 16 to 19° C. was the same as that observed in animals kept at 31° C. Denervated muscles of fasting animals underwent a more rapid loss of weight than muscles from fasting animals, or from denervated muscles alone. The total weight loss, however, was less than the sum of losses from control muscle during fasting, and by denervated muscles in animals on adequate caloric intake. Hines and Knowlton (97, 99) interpret these data as indicating that whereas fasting results primarily in a loss of the storage fractions of muscle, denervated muscles appear to lose storage fractions, and those fractions concerned directly with contractility. In addition, the denervated muscles lose the property of anabolic synthesis, thus contributing further to the rate at which weight loss occurs.

That weight loss in denervated skeletal muscle is not related to the phenomenon of fibrillation exhibited by these muscles, is shown by the work of Solandt and Magladery (200). The fibrillation may be prevented by adequate amounts of quinidine. In the absence of fibrillation the atrophy was still marked, and occurred at the same rate as that in denervated muscles in the absence of quinidine. Results of studies on the atrophic process indicated that the progressive loss of weight in denervated muscle is directly proportional to the lapse of time after denervation. According to Clinton and Hines (40), the relationship of the rate of weight loss in atrophy following denervation may be defined by the equation:

$$k = \frac{1}{t} \log_{10} \frac{A}{A - x}$$

when  $k$  is a constant characteristic of nerve viability, and  $A$  is the per cent of the original weight which may be lost at time  $t$ . Weight loss in denervation atrophy may proceed to about 20%, without loss of strength per unit muscle-cell phase (119). At this point a progressive and marked loss in the remaining muscle-cell

phase was found to occur. Atrophy from tenotomy was accompanied by severe impairment of the strength of muscular contraction. However, the change was found to be reversible following spontaneous repair of the tendon. The authors conclude, therefore, that a major factor in the maintenance of the contractile strength of muscle is the stretch produced normally by the attachment of the muscles to the bony skeleton. This process, they point out, is further enhanced by postural and by gross-movement reflexes.

Although in muscular atrophy due to peripheral or upper motor neuron section, or to tenotomy, there was considerable loss in weight of affected muscles after ten days, no change was then evident in the contractile power of the muscle per unit weight. There was, moreover, no detectable change in birefringence (64). Within three to five weeks, however, the contractile power and birefringence were distinctly diminished in muscles undergoing atrophy from denervation. In muscles atrophying due to upper motor neuron lesions and tenotomy on the other hand, no important changes were observed in contractile power and birefringence. Repeated electrical stimulation of the gastrocnemius of rats resulted in an increase in their weight and total isometric strength, but did not alter their contractile power. In denervated muscle there was close agreement between the loss in weight and isometric strength during the first ten days of atrophy. At the end of this early period of atrophy there was approximately 30% loss in muscle weight. During the later phase of atrophy the loss in tension was considerably greater than the corresponding weight loss (63). At this time there began to be appreciable loss in birefringence, which diminished at about the same rate as the contractile power per unit weight of muscle. The contractile power of muscle was therefore seen to parallel closely the birefringence throughout the period of atrophy. In normal muscle, according to Fischer (63), there exists a statistical correlation between birefringence and contractile power which can be expressed by the equation:

$$\text{birefringence} = k \times \log \text{contractile power}$$

Data on atrophic muscle, therefore, according to Fischer, supply further support to the hypothesis that the submicroscopic crystalline structure of muscle is closely related to the contractile mechanism.

### III. Muscular Hypertrophy

Few studies have been made of the chemical changes occurring in muscle as a result of hypertrophy. Most of these deal with cardiac muscle,

which appears to be the tissue of choice for such studies. Results of significant studies of chemical changes occurring in the rabbit heart during hypertrophy have been reported by Hitchings, Daus, and Wearn (102). Results of studies of certain chemical constituents of the rabbit heart were made at intervals following the rupture of an aortic leaflet. Such hearts undergo rapid hypertrophy, and chemical differences have been found between the constituents of muscles from hypertrophied hearts and those from normal myocardium. Some of these changes undoubtedly occurred as a result of a decreased concentration of capillaries in the hypertrophied myocardium, with consequent lengthening of the pathways for the diffusion of nutrients and metabolites (197). Data are available which indicate that within a short time after the production of experimental aortic regurgitation, the myocardium takes up considerable amounts of sodium chloride and water. An increase in the extracellular phase of heart muscle amounting to 10% has been reported following a brief ligation of the coronary artery (90). This is believed to have resulted from an increase in interstitial fluid and increased permeability due to injury. In the early stages of aortic insufficiency, it appears that the increase in extracellular phase of the myocardium results chiefly from an increase in interstitial fluid, rather than injury, since the intracellular elements undergo rapid growth, and concentrate potassium as rapidly as would be required by the synthesis of new intracellular material. The increase in extracellular fluid and other chemical changes in early hypertrophy were much the same as those reported to occur in other tissues when stimulated to rapid growth by means of certain hormones (130, 207). These data suggest that a similar pattern of chemical changes occurs in the rapid growth of tissue, regardless of the nature of growth stimulus. The most striking differences found between muscle from normal and hypertrophied hearts during the intermediate period (1-3 months postoperative) were the higher concentration of the intracellular phase and intracellular water of the hypertrophied tissue (102). The increased intracellular phase gave rise at times to greater than normal concentration of intracellular constituents when expressed per unit of tissue. This was particularly true of potassium, which in early hypertrophy was found to be increased. Later, the phenomenon is obscured by a loss of potassium which is characteristic in hypertrophy of long duration. It is possible that the relative increase in intracellular phase which accompanies hypertrophy may account for the rise in creatine concentration reported to occur in hypertrophy of less extent (95). After long periods of hypertrophy (4 months or longer) the myocardium tends to lose intracellular constituents, chiefly potassium and phospho-

lipide. The intracellular water content, however, remains elevated (102). Relatively marked losses of creatine and acid-soluble phosphorus occur after yet longer periods of hypertrophy (95). Hypertrophy and myocardial insufficiency in the human heart is likewise accompanied by loss of creatine, potassium, and phosphorus, and by an increase in extracellular electrolytes and water (32, 94, 164).

#### IV. Degenerative Changes in Muscle Resulting from Deficiency in Vitamin E

The first suggestion that substances designated as vitamin E play an indispensable role in the metabolism of skeletal muscle was contained in a report by Evans and Burr, in 1928 (57), when they showed that suckling offspring from rats deprived of vitamin E develop paralysis during the end of the nursing period. Lipschutz (128) in 1936 described widespread degenerative changes in the central nervous system in tissue from animals deprived of vitamin E which Evans and Burr placed at his disposal. It was Olcott (171), however, who discovered that the paralysis in these rats was due to degenerative lesions in the voluntary muscles. Olcott could find no lesions in the central nervous system. In the meantime, Goettsch and Pappenheimer (72), as early as 1931, had described profound lesions in the muscles of rabbits and guinea pigs on diets which had been treated with ethereal ferric chloride, which we now know resulted in the destruction of vitamin E. At the present time, evidence is abundant that the selective necrosis of muscle fibers seen in this deficiency syndrome is due to a lack of vitamin E in the diet, and that vitamin E, in its natural form or as synthetic  $\alpha$ -tocopherol, prevents or cures the disease in guinea pigs and rabbits (55, 135, 195). Vitamin E deficiency in dogs has also been produced and is likewise corrected by  $\alpha$ -tocopherol (5).

Results of studies on the pathology of the muscles in nutritional muscular dystrophy do not permit definite conclusions as to the pathogenesis of the lesions. According to Pappenheimer (173), the changes seen in the muscles could be brought about by excessive contraction of the fibers with subsequent rupture and necrosis; by a direct and selective toxic action on the muscle cells; or by arteriolar occlusion, with subsequent infarction and anoxia. The fact that muscle fibers which receive a direct and abundant supply of blood from the intermuscular fascia generally escape necrosis is cited in favor of the latter hypothesis. Such fibers are favored metabolically over more remote areas of muscle, and might be expected to yield last to the effects of vitamin E deficiency. According to Pappenheimer,

the first stages of the disease occur "with explosive suddenness." The pathology of later stages of the disease, on the other hand, is one of reaction to necrotic tissue and of active regeneration. It is thus possible to estimate the duration of symptoms with reasonable accuracy from an inspection of the muscular tissue.

The pathology of muscle fibers undergoing degeneration from vitamin E deficiency is fairly specific (174). In well-advanced dystrophy as many as 50% of the fibers, which are widely separated by edematous stroma, show hyaline necrosis with segmentation and rupture. In the stroma are large numbers of elongated fusiform myocytes showing vesiculated nuclei and basophilic cytoplasm. There are also many histiocytes and mature lymphocytes. Changes are seen in the muscle of embryos showing dystrophy similar to those recorded in certain stages of adult nutritional muscular dystrophy. The heart is apparently unaffected histologically (55). According to Knowlton *et al.* (120), the histochemical changes in the muscles of animals deficient in vitamin E can be detected well in advance of generalized symptoms (137). These changes consisted in a measurable increase in the concentration of water and chloride, a decrease in creatine concentration, and in maximal strength. There were accompanying focal areas of hyaline necrosis. In well-developed dystrophy there were marked reactive inflammatory changes and beginning regeneration of muscle fibers. Intact fibers showed an increase in the number of sarcolemmal nuclei.

A number of studies have been made of the physiological and chemical changes which occur in muscle undergoing degeneration in vitamin E deficiency. It should be emphasized, however, that interpretation of these changes is very difficult, owing to the varying composition of muscle under these conditions, particularly with respect to the degree of necrosis, number of regenerating fibers, amount of interstitial connective tissue and fat, and the degree of calcification occurring in dead muscle fibers.

A decrease in total creatine content of the muscle in nutritional dystrophy has been found consistently. The decrease is roughly proportional to the degree of fatty and fibrous infiltration (70). The per cent of creatine in esterified form, however, is reported to be considerably greater than normal (157). Doubtless much of the decrease in creatine concentration is due to reduction in the number of normal fibers. However, reduction of creatine has been reported in muscles showing only necrosis, without fibrosis or fat replacement. In all muscles examined, the creatine content was found never to fall below that normally present in the heart. In human muscle, undergoing degenerative changes in a variety of syndromes, the lowest values for creatine were likewise equal to that found in

the heart (23). Goettsch and Brown feel that this may be a coincidence, or may indicate that creatine is bound in more than one way in striated muscle (70). White muscle showed a greater reduction in creatine than red muscle. White muscle also showed more extensive pathological lesions. No change in the creatine content of heart and brain were recorded even in the last stages of degeneration in the skeletal muscle (70). The moisture content of degenerated muscle with normal fat concentration was higher than that of normal muscle. A gain in sodium chloride and calcium and a corresponding loss in potassium and magnesium in the muscles in nutritional dystrophy have been reported by Fenn and Goettsch (59) and by Morgulis and Osherhoff (156). The glycogen content of skeletal muscle in nutritional dystrophy is greatly reduced, with the reduction proportional to the degree of dystrophic change.

Results have been obtained for the rheobase and chronaxie of dystrophic muscles at various stages of degeneration in vitamin E deficiency (220). In all cases, both the rheobase and chronaxie were found to be greater than normal, with the former considerably increased over the latter.

Results of studies of changes in chemical constituents of the muscles in rabbits with vitamin E deficiency have been reported by Morgulis *et al.* (159). Hypercholesterolemia is an outstanding characteristic of rabbits affected with nutritional muscular dystrophy. The cholesterol content of the skeletal muscles is likewise considerably increased (159). Evidence has been presented which is interpreted as showing that the marked increase in cholesterol which occurs in the muscles of dystrophic animals, results from synthesis and not from redistribution of pre-existing cholesterol. Of the tissues studied, only skeletal muscles showed this increase. There was also a marked increase in total fat and lipid phosphorus. No changes in the concentration of lipoids were found in the tissues of the heart and other internal organs. In advanced dystrophy the lipid content of the muscles was often doubled, while the cholesterol concentration was found to increase from 100 to 350% of that found in skeletal muscle of control animals. The phosphorus fractions in nutritional muscular dystrophy in rabbits have been studied by Goettsch and associates (71). No striking changes were found in the phosphorus fractions until the muscles showed severe degeneration. At this time, there was a marked increase in total, total acid-soluble, and total inorganic orthophosphate phosphorus associated with calcification of the fibers. Muscles which did not show calcification were found to have a decreased concentration of these constituents. The phosphocreatine content of degenerated muscles, in the resting condition, was distinctly lower than normal, but there was no change



in its relation to total acid-soluble phosphorus. No changes were found in the phospholipide content.

Respiratory studies on the skeletal muscles of dystrophic animals have produced some very interesting results. In nutritional muscular dystrophy of rabbits, the oxygen consumption of diseased muscles was found to be increased as much as 400% above the mean of the normal rate of control tissue (66, 220). Results of studies of the influence of  $\alpha$ -tocopherol on the gaseous exchange of muscle in nutritional dystrophy have provided us with the first genuine clues as to the possible mechanism of action of this vitamin. Studies of biopsy material showed that the parenteral administration of  $\alpha$ -tocopherol phosphate resulted in a drop in the consumption of oxygen by dystrophic rabbit muscle of approximately 35% in the first hour (108). Thereafter the fall in oxygen consumption continued during four hours to a nearly normal figure. A sharp decline in the creatine concentration of the muscles accompanied the lowering of oxygen consumption during the first two hours, with a slight rise at the end of four hours. The chloride concentration of the muscle remained high throughout, and was not immediately influenced by  $\alpha$ -tocopherol.  $\alpha$ -Tocopherol had negligible effects on normal muscle, except for a slight increase in the oxygen consumption after four to six hours.

Of the various animals studied, muscle from dystrophic hamsters showed a striking increase in oxygen consumption, with figures recorded as high as 250% of the normal (109). The oxygen uptake appeared to increase with the severity of the dystrophic condition. The effects of  $\alpha$ -tocopherol in lowering the  $Q_{O_2}$  of dystrophic muscle *in vitro* have also been recorded (107). The high oxygen consumption of slices of dystrophic rabbit and hamster muscle was lowered 40%, and in some cases to values which were near the normal, by the addition of  $\alpha$ -tocopherol phosphate to the medium. No influence of  $\alpha$ -tocopherol could be detected on the  $Q_{O_2}$  of normal muscle slices. The  $Q_{O_2}$  of boiled muscle slices was negligible, and was not influenced by the addition of  $\alpha$ -tocopherol phosphate. The succinoxidase activity of the dystrophic hamster muscle was 160% higher than that of normal muscle, and was sensitive to the addition of malonate.  $\alpha$ -Tocopherol phosphate lowered the succinoxidase activity of dystrophic muscle by 59%, which was comparable to the decrease in the  $Q_{O_2}$  of muscle slices when  $\alpha$ -tocopherol phosphate was applied *in vitro* or administered *in vivo*.

It is becoming increasingly evident that it is impossible to reconstruct to any marked degree the chemical events in the tissues in disease, from a study of the changes which occur in the constituents of blood and urine.

However, clues to the nature of aberrant processes in the tissue are sometimes uncovered, and for this reason such studies are important. Glycemic curves, along with other studies on the blood and urine, have been determined in rabbits with nutritional muscular dystrophy by several investigators. The glycemic curve is reported to differ from the normal in that it rises more slowly, reaching a peak in about twice the length of time. It does not attain as high a level as the normal and slopes off more gradually. Analyses of fasting blood from normal and dystrophic rabbits do not indicate any marked variation between the two groups as far as glucose, lactic acid, total acid-soluble phosphorus, and its various fractions are concerned (157). Blood cholesterol and lipid phosphorus show a marked increase in dystrophic animals, the increase being approximately proportional to the degree of dystrophic change. With change to normal diet, or when supplements of vitamin E are administered, the changes are reversed, the level returning ultimately to normal.

The metabolism of creatine and creatinine has received special attention in studies of muscle degeneration in vitamin E deficiency, although studies of the most diverse variety have been described. During the period of approaching dystrophy, the reaction of the urine was found to become increasingly acid (158). The urinary chlorides decreased, while elimination of phosphate increased. There was some decrease in the excretion of total nitrogen. The most marked change in urinary excretion, however, was in creatine, which was at times more than doubled. No appreciable decrease in creatinine occurred, however, until a condition of extensive atrophy had occurred. Finally the body weight began to drop sharply. At this point there was marked diuresis, accompanied by a slight increase in creatinine and sufficient increase in total nitrogen to produce a definitely negative nitrogen balance. Near the end stages of the deficiency, according to Morgulis and Spencer (158), there was a definite decrease in urine volume and in the excretion of creatinine. The excretion of creatine, however, remained high until death. Terminally there was again a period of diuresis. During the period of recovery, the weight at first increased slowly, followed by an accelerated gain in weight, and the appearance of a normal concentration of urinary phosphorus, chloride, nitrogen, creatine, and creatinine.

## V. Diseases of Voluntary Muscle in Man

Few syndromes are as confusing to the biologist as the primary muscular disorders in man. Much of the confusion is due to lack of agreement on the clinical and physiological findings in these affections. The state of con-

fusion is further enhanced by the fact that although this group of diseases has been recognized as clinical entities for half a century or more, the pathogenesis has remained obscure. A metabolic attack on the problem has yielded certain information, but the data are contradictory, and thus far most attempts at interpretation have been sporadic and unsatisfactory. It is becoming increasingly apparent, however, that no sharp lines can be drawn between many of the primary syndromes of muscle, and that some unknown deficit in metabolism is responsible for the biological continuity and sequence of phenomena common to many of these clinical disorders.

In Table I taken from a more complete table compiled by Aring and Cobb (8) are listed the primary disorders of muscle, as described and classified in modern textbooks of neurology and medicine. Excellent reviews on the over-all descriptive anatomy and pathophysiology of these diseases have been written by Aring and Cobb (8) and Nevin (168). Only the more significant results which are available from studies on the metabolic aspects of these diseases will be covered here.

TABLE I  
CLASSIFICATION OF THE PRIMARY MUSCULAR ATROPHIES ACCORDING TO MOST  
TEXTBOOKS OF NEUROLOGY\*

Sporadic	Familial	
	Myopathic	Myelopathic
Myasthenia gravis	Family periodic paralysis	Amyotonia congenita
Progressive muscular atrophy	Progressive muscular dystrophy	Infantile muscular atrophy
Amyotrophic lateral sclerosis	Myotonia	Hypertrophic neuritis
	Dystrophia myotonia	Peroneal muscular atrophy
		Familial ataxia

\* Taken from chart by C. D. Aring and S. Cobb (8).

### 1. *Myasthenia Gravis*

Myasthenia gravis is a fatal disease characterized by progressive fatigability and weakness of the muscles, especially those of the neck, lips, throat, tongue, face, and eyes. Early in the disease attacks of weakness and fatigability are transitory, but as the disease progresses less and less exertion is required to produce muscular paralysis, and the attacks persist for increasing periods of time. Spontaneous remissions occur in about 25% of cases. The first description of myasthenia gravis was given by Thomas Willis in his *De Anima Brutorum* published in 1672 (209). No significant pathology has been found in the nervous system. Examination of the muscles frequently reveals deposits of lymphoid material in ill-defined areas between muscle fibers, and particularly around capillary vessels (24). The thymus gland is enlarged or otherwise abnormal in about half the

cases (8, 153, 163). Occasionally there are pathological changes in the thyroid gland (121, 214).

The search for the mechanism underlying the syndrome of myasthenia has been greatly stimulated in recent years by the development of knowledge of the neuromuscular transmitting apparatus. The discovery by Walker (224) that prostigmine restored normal motor power to myasthenic patients, together with other physiological and pharmacological studies, suggested the hypothesis that in myasthenia gravis there is defective neuromuscular transmission. The fact that prostigmine is a strong inhibitor of the enzyme cholinesterase, brought into association with studies of this disease certain fundamental work of Loewi (129) and Dale (45) on the pharmacology and metabolism of acetylcholine, and further suggested that the faulty neuromuscular transmission was linked to excessive destruction of this substance by cholinesterase in serum and muscle. In theory, at least, myasthenia gravis might be related to an inadequate release of "transmitter substance" at the neuromuscular junction, to a high threshold of excitability at the motor end plate, or to an exceptionally high rate of destruction of the transmitter substance (38).

Numerous studies have been made on the metabolism of acetylcholine in myasthenia gravis on the assumption that it represents the transmitter substance. Milhorat (146) has secured results which he interprets as rendering unlikely the theory that in myasthenia there is an excessive destruction of acetylcholine due to large amounts of cholinesterase; while Lanari (122) and others (86, 88), have performed studies, the results of which they feel have disproved the hypothesis that in myasthenia gravis there is a defect in muscle which results in inadequate utilization of acetylcholine. Torda and Wolff (215) lean to the hypothesis that there is defective synthesis of acetylcholine in myasthenia gravis, and have published results which they interpret as showing a decrease in the synthesis of acetylcholine by the sera of patients with myasthenia, when mixed with frog brain preparations. According to these investigators, the decrease in synthesis was specific for myasthenia, since it was not encountered in other diseases associated with debility, cachexia, and prostration. Stoerk and Morpeth (204), on the other hand, using rat brain preparations, failed to observe significant differences between the amounts of acetylcholine formed by the addition of normal serum and serum from patients with myasthenia gravis. Torda and Wolff (216) in a subsequent communication suggest that rat brain suspension may not provide a system sensitive enough to detect differences between the synthesis of acetylcholine in the presence of sera from normal and myasthenic subjects. In this com-

munication, moreover, they state that human spinal fluid is a more favorable medium than serum for the demonstration of the synthesis of acetylcholine in the presence of a suspension of frog brain.

Owing to difficulties encountered in securing specimens of human muscle for comparative studies of cholinesterase activity, most workers have resorted to the use of serum. However, a few results have been reported for human muscle by Jones and Stadie (113) and Gilman *et al.* (74). No significant differences were found by these workers between the cholinesterase activity of muscles from myasthenic patients and that from control subjects. Jones and Stadie state, moreover, that in studies on the effect of prostigmine on muscle cholinesterase, they were unable to show any action of prostigmine *in vitro*, at approximately therapeutic concentration, upon the esterase activity of muscle from human subjects and guinea pigs. However, similar concentrations resulted in a marked decrease in the esterase activity of the serum. The hypothesis, therefore, that myasthenia gravis is due to an excess of cholinesterase in muscle, and that this activity can be depressed by prostigmine, receives no support from their data.

With few exceptions, workers have reported normal values for the cholinesterase activity of the serum in myasthenia gravis. Stedman (202) and Stedman and Russell (203) reported values for myasthenia patients which were lower than normal, and thereby concluded that myasthenia was not due to excess cholinesterase. Goodman *et al.* (74) reported an abnormally high value for cholinesterase on one patient. McGeorge (134), Hall and Lucas (81), Russel *et al.* (184), Milhorat (146), Poncher and Wade (176), and, more recently, Wilson and Stoner (227) have reported values for serum cholinesterase well within the normal range.

Attempts to demonstrate that the concentration of acetylcholine liberated at the myoneural junction is less than normal in myasthenia gravis, failed because of obvious technical difficulties. Attempts to produce clinical relief of myasthenia by the subcutaneous injection of acetylcholine (43, 223) have likewise met with failure. Fraser *et al.* (65a), however, with huge doses of acetylcholine (500–600 mg. subcutaneously), claimed to have produced recovery in muscular power which was sustained for several hours. Harvey and Lilienthal (86) injected acetylcholine into the brachial artery of myasthenic patients, and produced involuntary flexion of the fingers, hand, and wrist, but were unable to obtain sustained improvement. They concluded that since the muscle fibers in myasthenia were capable of vigorous contraction when adequate stimuli were applied, there could be no primary defect in muscle, but rather that the defect was in the nerve or neuromuscular conduction apparatus.

Nevin (166-168) and other investigators (14) from time to time have suggested the possibility that there exist in the blood and tissues of myasthenic subjects, metabolites with curare-like action which raise the threshold response of the neuromuscular junction to the action of acetylcholine. Walker (224) claimed that, by occluding the circulation in the upper limbs and exercising the muscles of the forearms, there was produced, following the release of constriction, an increase in the clinical signs of myasthenia after a short latent period. Wilson and Stoner (227) have recently repeated the experiments of Walker, using a cine-camera to record changes in ptosis and in the state of the muscles of expression. Increased ptosis was noted in eleven cases out of fourteen. The observations were interpreted as confirming the results described by Walker. Blalock *et al.* (17), on the basis of beneficial results obtained in myasthenia gravis by thymectomy, have suggested that the block at the motor end plate in this syndrome may be related to a substance released by the thymus. Further evidence that the block may be due to a humoral substance has been provided by Wilson and Stoner (227), who have recently reported that the serum of patients with myasthenia gravis produced a block in neuromuscular transmission when tested on isolated nerve-muscle preparations of the frog.

Significant results of studies on the abnormality of neuromuscular transmission in the hands of patients with myasthenia gravis have been published by Harvey and associates (87). In three patients it was found that a single maximal stimulation of the nerve was sufficient to excite a portion of the muscle correspondingly innervated. In one case facilitation in transmission resembling that seen in partially curarized animals was observed. In yet another case, depression in transmission following passage of a single-volley stimulus was so severe that it could not be restored to normal with prostigmine. From these data, Harvey *et al.* (87) concluded that the changes in action potential in myasthenia arise as a result of events which take place at the neuromuscular junction.

Myograms made from patients with myasthenia gravis are said to be characteristic and abnormal when obtained at high rates of stimulation of the motor nerve (169). The myograms became normal, however, within 45 minutes after the intramuscular injection of prostigmine. The action with prostigmine was immediate when the drug was given intra-arterially.

Few results have been obtained on the chemical constituents of muscle in myasthenia gravis, aside from those cited for the cholinesterase content. Nevin (166) has studied the phosphorus-holding compounds of myasthenic muscle, and has reported values well within the normal range. A slight

increase in the concentration of soluble carbohydrate esters was not considered of significance. From these data it has been concluded that the disease is not associated with an abnormality in the intrinsic chemical mechanism of muscular contraction. Moreover, following electrical stimulation of the muscle, the changes in distribution of organically bound phosphorus were qualitatively and quantitatively that seen in normal muscle treated to the same strength of stimulus. Reversal of these changes with rest was as nearly complete as in the case of normal muscle.

Many reports have been given purporting to show that there are various types of metabolic aberration in myasthenia gravis. Early reports dealt with claims that there was a marked loss of calcium from the tissues (48), occasionally associated with nitrogen retention (175). Marburg (140) reported values for blood magnesium concentration twice that found in normal subjects, and advanced the theory that it resulted from thymus hyperactivity associated with myasthenia. Reuter and Zimmermann (182) reported a low alkali reserve after exercise which they claimed was associated with the maintenance of a high postexercise level of blood lactic acid. They did not, however, think the changes were specific, but rather secondary to other phenomena associated with the myasthenia syndrome. Results obtained by others (91, 92, 181), and particularly those obtained by Boothby and associates (1), are, in general, contradictory to the idea that significant changes are found in blood constituents in myasthenia gravis. Results obtained by these investigators showed normal values for blood calcium, magnesium, sodium, potassium, and phosphorus. Moreover, normal values were reported for blood sugar, urea, creatine, amino acids, and uric acid. It was their impression that results of balance studies on minerals and nitrogen which they carried out, as well as those obtained by other investigators, indicated that the immediate condition of the patient with myasthenia gravis determined the type of balance obtained, rather than that any particular type of aberration was characteristic of this syndrome.

Contrary to certain previous reports (48, 145), Milhorat and Wolff (152) and Boothby and associates (1) found no striking abnormalities in the excretion of creatine or creatinine in myasthenia gravis except for moderate increase in excretion of creatine during exacerbations of the disease. According to these workers, the fundamental metabolic disturbances which occur in myasthenia are not centered in the metabolism of creatine and creatinine, but the metabolism of creatine is involved secondarily when the disease becomes so severe that the prognosis for life is very grave.

## 2. *Myotonia*

The condition myotonia, often referred to as Thomsen's disease (213), is a syndrome in which a state of muscular contraction occurs when the patient attempts voluntary movement after resting. Not only is there subjective disability of limitation of movement, but there is likewise an objective phenomenon of sustained contraction resulting from mechanical or electrical stimulation (56). The pathological defect which is responsible for delay in relaxation of the muscles has been supposed by some to be inherent in the musculature, and by others to be associated with some basic alteration in the central nervous system. Proof of the presence of delay in a "nerve-muscle preparation" was provided by Grund (78) and Schäffer (187), who demonstrated delay in relaxation of muscles in patients with myotonia when the limbs were paralyzed by intrathecal injection of local anesthetics. Available evidence suggests that myotonia, like myasthenia gravis, is due to an abnormality of the apparatus concerned with neuromuscular transmission, except that the excitability would be lower in myasthenia gravis, and higher in myotonia than in the case of normal muscle (85). It has been suggested that the delay in relaxation in myotonia is due to an excessive liberation or abnormal accumulation of acetylcholine at the motor end plates, but no evidence supporting this hypothesis has been obtained (185).

According to Denny-Brown and Nevin (47), the mechanism of delay in muscular relaxation in patients with myotonia is a complex phenomenon, in which the muscle fibers when excited lead "to repeated discharge of the action-current mechanism of the muscle fiber." It is suggested that this may be an exaggeration of a normal component of the mechanism of muscular contraction in which there occur subsiding excitations in muscle fibers following one nerve impulse (53). Denny-Brown and Nevin further suggest that this phenomenon of high-frequency discharge reflex in "after-spasm" in muscles may be responsible for hypertrophy of muscle fibers which are seen in this disorder. They suggest, moreover, that it would be desirable for the purposes of elucidation of the true nature of the disorder "to clear away the central nervous system complications of this affection in order to arrive at a proper conception of both its true mechanism and morbid histology."

A number of studies have been made on the metabolic state and composition of the blood of patients with myotonia. In most instances, the results have been normal, or when abnormal their specific relationship to myotonia has been held in question (179). Values for blood calcium have been normal in most reports. Blood sodium and potassium (79), bromide



and iodide (6) have been determined in patients with myotonia and normal values recorded. The alkali reserve is said to be slightly increased (6). The basal metabolic rate has been reported to be low in myotonia of the dystrophic variety, but the condition was not improved by the administration of thyroid (179). Numerous studies of creatine excretion in patients with myotonia have been made, but no convincing relationship has been established between an aberration in creatine metabolism and myotonia, except in those conditions in which hyperthyroidism is an associated phenomenon (176, 177). Values for resting blood lactic acid have been reported to be high by Wassermeyer and Dutte (225), and as being unchanged by Friesz and Mohos (67).

The administration of quinine to patients with myotonia affords some improvement in diminishing delay in muscular relaxation (80, 116, 199). The fact that quinine has a curare-like effect, in that it decreases excitability of the motor end plate, has led to the hypothesis that this is the mechanism by which the drug accomplished improvement in myotonia (85).

Few convincing reports have been made on the gross and microscopic pathology of the muscles in myotonia. The muscles are said to be slightly paler than the normal. The muscle fibers show rounding and hypertrophy, with poorly marked transverse striations. The nuclei of the sarcolemma may be increased. No changes are found in the central or peripheral nervous system (8, 42).

### 3. *Familial Periodic Paralysis*

Family periodic paralysis was first described by Cavaré, in 1853 (36). Clinically the disease is recognized by the occurrence of sudden periodic attacks of flaccid paralysis, with diminution or loss of excitability in the muscles of the affected limbs, and by marked depression or absence of deep reflexes. The attacks vary widely in severity from a state of partial paralysis of one limb to a condition of complete involvement of all the muscles of limbs and trunk and, at times, including certain muscles innervated by the cranial nerves. The condition may pass in a few hours, or it may remain for several days. In exceptional cases death may occur at the height of the attack (106). The disease is frequently associated with hyperthyroidism (197), and reports of marked improvement (161) and cure of the disease in some cases (51) have been reported to follow relief of this condition.

Excellent reviews of the clinical and pathological aspects of the disease have been written by Taylor (210), Singer and Goodbody (198), Mitchell (154), Westphal (226), Goldflam (73), and Talbott (208).

Results of studies purporting to show specific pathological changes in the muscles in this disorder are not very convincing. Goldflam (73) has described dissociation and degeneration of the fibers, with an increase in caliber and vacuolation. No evidence of interstitial inflammatory changes of muscle fibers, or changes in the nervous system have been convincingly demonstrated. Subsequent reports on biopsies of muscle in this disease have been entirely negative with respect to pathological changes. Thus it is quite likely that changes previously described were artifacts (136). Periodic paralysis is often familial, but there have been many cases in which no hereditary basis was established. There is a strong predilection of the disease for males over females, in a ratio of approximately 3:1 (208).

Although family periodic paralysis had been under investigation for over fifty years, the mechanism by which the paralytic attacks are produced remained completely obscure until results of studies reported by Aitken and associates (2) and Gammon (68), who called attention to the relation between the serum potassium level and the development of muscular weakness in this syndrome. A low value for blood potassium and lactic acid, together with lowered values for phosphate, had been previously shown in one case of family periodic paralysis, but no special significance had been attached to the findings (16). As early as 1902 the oral administration of potassium citrate was believed to have some favorable effect on seizures in periodic paralysis (155), and more recently Herrington (93) stated that attacks could be prevented entirely if potassium salts were given before the onset of the seizure. He did not, however, think that the administration of potassium would end an attack when already induced. It was these observations and the fact that substances such as adrenaline, insulin, and sugar, which are poorly tolerated in periodic paralysis, and which are now known to lower the concentration of potassium in the serum (196, 228, 229), which led to studies on the metabolism of potassium in family periodic paralysis.

During severe attacks of family periodic paralysis, the potassium in the serum may be lowered 25 to 30% (68). Muscular weakness was observed when the serum level of potassium fell to 12 mg. per cent. Levels of 10 mg. per cent or lower were associated with a full attack. A diminution in the serum potassium level during attacks is associated with a diminution in the urinary excretion of sodium, potassium, fixed bases, and chlorides (208). When sufficient quantity of potassium chloride was ingested by the patient to raise the level of serum potassium past 12 mg. per cent, muscular power was observed to return, with subsequent relief of the attack (144). According to Gammon (68, 69), seizures are most likely to occur

early in the morning, when the absorption of potassium from the intestinal tract is low, and when the potassium ingested with the day's diet has been largely eliminated. In Gammon's case oral potassium chloride was effective in relieving attacks in the presence of normal serum potassium concentration. Other salts, such as sodium chloride and sodium bicarbonate, were entirely ineffective. Creatine hydrate and prostigmine also failed to diminish weakness. It is not clear if a low concentration of potassium in the serum is in itself sufficient to produce paralysis in susceptible subjects. It is possible that the neuromuscular apparatus itself may be abnormal (16). This hypothesis would explain the fact that paralysis in this syndrome is frequently confined to the muscles of the limbs and trunk. An alternative explanation would require that certain muscles are more sensitive to lowered levels of serum potassium than others. In line with the idea that there may be active pathological involvement of the nerve or muscles, is the observation that atrophy and wasting of musculature is occasionally associated with severe and prolonged manifestation of the disease (2).

The mechanism by which a lowered level of potassium in the tissues and blood produces paralysis is not clear at the present time. Aitken *et al.* (2) propose possible interference with one or more of three processes: (1) the passage of the nerve impulses along the fibers of the nerve; (2) its transmission across the neuromuscular junction; (3) the response of the muscle fiber to the impulse at its motor end plate.

Although a decrease in the concentration of potassium of the blood and tissues appears to be intimately related to paralysis in susceptible individuals, a diminished concentration of potassium in the serum may be observed in susceptible, as well as in nonsusceptible subjects without objective or subjective evidence of paralysis (3, 208). Serum potassium levels in man lower than 10 mg. per cent have been observed without paralysis following the injection of insulin. Potassium values as low as 8.5 and 7.2 mg. per cent have been found in diabetes with no mention of paralysis (84, 117). In the case of periodic paralysis reported by Ferrebee and associates (60), spontaneous attacks were accompanied by a temporary decrease in the serum potassium and almost complete disappearance of potassium from the urine. Since no exceptional increase in urinary potassium immediately preceded these attacks, it was reasoned by these workers that the sudden disappearance of potassium from the serum and urine was not due to depletion of stores of potassium from the body. The increased excretion of potassium which followed the seizure was more in line with the hypothesis that a transitory retention of this substance by the tissues had

reduced the concentration of circulating potassium. This alternate retention and release of potassium, which was observed to continue during absence of attacks of paralysis, was interpreted as showing that the abnormality of potassium metabolism in family periodic paralysis is not limited to the period of paralysis. Diminution in serum phosphate accompanies the diminution of serum potassium in attacks of paralysis. Frequently this is associated with a negative phosphate balance. In one case studied by Milhorat and Toscani (149), the phosphate balance was persistently negative for a period of 42 days. Significant amounts of phosphorus were lost, even when the diet was high in this constituent. The loss of phosphorus was believed to be chiefly from the muscles, since no change in the bony structure was observed, and the balances of calcium and magnesium were normal.

The immediate temporary fate of the potassium which disappears from serum just before, or at the onset of, an attack of periodic paralysis cannot be stated with certainty. Allott and McArdle (3) have calculated that in a spontaneous attack about 800 mg. of potassium which would normally be excreted is retained. There was a loss of about 1500 mg. from the extracellular fluids of the body when there was a fall of 10 mg. per cent in the serum, if an extracellular fluid volume of 15 liters was assumed. It seemed likely to these investigators that in periodic paralysis there is, for some reason, a periodic increase in demand for potassium in the muscles of affected subjects, and that the fall in extracellular potassium concentration and the diminution in the excretion of urinary potassium are secondary effects of an attempt to meet this demand. The approximate parallelism between the movement of potassium and phosphorus in metabolic studies on periodic paralysis suggests to these workers that some mechanism involving hexose phosphate is concerned. There is evidence in normal metabolism that potassium in the blood and tissues follows closely the movement of carbohydrate from muscle to liver and back again, and that it participates intimately in the carbohydrate cycle in the cells of the liver and muscle (58). This hypothesis receives some support from the observation that glucose, insulin, and adrenaline (2, 3, 178), all of which affect the metabolism of hexose phosphate and the movement of potassium in the tissues, are known to foster the development of attacks of paralysis in susceptible subjects.

Although there is increasing evidence to support the hypothesis that the site of the disorder in familial periodic paralysis probably lies somewhere in the periphery of the neuromuscular system, various organs from time to time have been indicated as being the primary site of the disordered mecha-

nism. Mankowsky (139) argued that the cause lay in a pathological change in the vegetative centers of the midbrain, due to an inherited defect in the regulators of the physicochemical state of muscle tone, and particularly with respect to the control of the migration of potassium and calcium. Moreover, he argued that paralysis was only an expression of an alteration in vegetative function, which is manifest also in changes in the circulatory system (flushing), and in the secretory system (sweating, etc.). Biernacki and Daniels (16) also leaned to the view that the disease was most likely the result of an alteration in the autonomic nervous system, and cited in support the fact that cold and emotional states were precipitating factors, and that, when induced, the attacks were accompanied by irregularity of the pulse, dyspnea, dysuria, and sweating. Bornstein felt that the disease had certain components in common with epilepsy (25). Yoshimura (228) believed that the condition was related to dysfunction of the parathyroid gland, and reported a high concentration of magnesium in relation to calcium in the blood of patients in support of his theory. Westphal (226) was of the opinion that the attacks of paralysis were precipitated by a spasm of the peripheral vessels resulting in a sudden disturbance of the nutrition of the muscles.

Few reports have been made on the chemical constituents of the muscle in subjects with periodic paralysis. Zabriskie and Frantz (230) report studies on biopsies of paralyzed and nonparalyzed muscles in one case. The oxygen consumption of the paralyzed muscle was reported to be strikingly lower than in the nonparalyzed control. Paralyzed muscle consumed 2.62 cc. oxygen per g. per min., and nonparalyzed muscle 5.3 cc. per min., while two specimens of control muscle from a normal subject consumed 9 and 8.4 cc. oxygen, respectively. Organic- and inorganic-acid-soluble phosphorus were considerably lower in the muscle specimen of the patient than in that of the normal control, although no striking difference was noted in the phosphocreatine content of the two types of muscle (27, 192).

#### 4. *Progressive Muscular Dystrophy*

Few syndromes among the diseases of muscle have provided more challenge to the clinical investigator than progressive muscular dystrophy. Original descriptions of this disease are credited to Meryon, 1852 (143), and to Duchenne, 1861 (50). Progressive muscular dystrophy, first clearly defined by Gowers (75, 76), is characterized by primary degeneration and atrophy of the voluntary muscles of the extremities, pelvis, and shoulder girdles. Atrophy is marked in all forms, although pseudohypertrophy is an early and prominent symptom in one form. In the commonest type,

pseudohypertrophic muscular dystrophy, the onset generally occurs before the fifth or sixth year. The disease has certain well-established familial aspects, although the mode of genetic transmission is not clear (13, 147). The prognosis is grave, and death frequently results within several years after the first symptoms of the disease become manifest. No specific therapy is known and little in the way of supportive treatment has proved useful.

Morphological studies on progressive muscular dystrophy have yielded little information aside from the fact that there is hyaline degeneration and fragmentation of the muscle fibrils, and that the muscles show marked increase in connective tissue and fat (89). It is to this increased fat concentration that the muscles seem to owe their excessive bulk. The recent development of a simplified quartz microscope, with the 2537 Å line of mercury as the light source, has made it possible to obtain ultraviolet photomicrographs of tissues fixed, embedded, and sectioned by routine methods (124, 125). This technique has been shown to have particular advantages in the cytological study of muscle when it is desired to correlate certain morphological and physiological changes (141). The appearance of unstained sections of muscle is often misleading, since the image which is obtained under these conditions is due to inhomogeneity of the tissue rather than to the presence of material with selective absorption (35). The technique of ultraviolet photomicrography has been applied with some success in attempts to demonstrate the chemical nature of the components of various tissues (33, 34). An extension of the technique of ultraviolet photomicrography to a study of the histological changes in progressive muscular dystrophy has been made by Hoagland *et al.* (104, 105). The specialized structure of muscle is such that it allows a fairly satisfactory comparison to be made between larger structures revealed in ultraviolet photomicrographs and those which are revealed by staining and photography in ordinary light. Photomicrographs made by ultraviolet light depict a variety of histological changes associated with various stages in the development of progressive muscular dystrophy, which appear to be specific for the disease. In moderately advanced progressive muscular dystrophy, it was observed that portions of the muscle fibers had been replaced by fat and fibrous connective tissue. The main outline of the remaining fibers was well preserved, although the endomysium appeared friable, and, unlike normal muscle, suffered considerable fragmentation when cut with the microtome. There was marked variation in the appearance of the sarcolemma, with areas which showed intense absorption and others in which the absorption was so slight as to indicate that com-

plete interruption in continuity of structure had occurred. The transverse zones showing selective absorption were thin and absorbed with less intensity than the broad, compound bands which were observed in transverse sections of normal muscle. A study of sections of dystrophic muscle in the prefibrotic stages, revealed that although the muscle showed absorption as intense as that of normal muscle, the absorbing areas were no longer well defined, and areas of degeneration and fatty infiltration appeared in many of the fibers. It was possible, therefore, by means of ultraviolet light to detect changes in the muscles of subjects with progressive muscular dystrophy long before they were revealed by other techniques of examination.

Notwithstanding the fact that progressive muscular dystrophy has engaged the interest of clinicians since the original description of the syndrome in the middle of the 19th century, almost no attempts were made to study the general metabolism in this disease until the pioneer work of Levene and Kristeller in 1909 (127). These workers showed that the feeding of protein resulted in an excessive excretion of creatine in patients with progressive muscular dystrophy. Subsequently the results of many studies have revealed that there is marked derangement in the metabolism of creatine in this disease (148, 212), and that endogenous creatine, formed from proteins and amino acids, is not retained by the muscles as effectively as in normal subjects. When creatine is ingested by patients with progressive muscular dystrophy, much of it is excreted in the urine, the amount retained depending largely on the severity of the disease (28, 150, 151). This observation has led to the concept in progressive muscular dystrophy of a "diabetic-like state" with respect to the ability of the patient to retain either ingested creatine, or creatine formed endogenously from proteins and amino acids. Whether or not this is a true concept, the recognition of the biochemical aberration in creatine metabolism is perhaps the most significant contribution to have been made toward an understanding of the essential nature of this disease within the past thirty years. The diminished excretion of creatinine is perhaps of greater significance in progressive muscular dystrophy than the increase in excretion of creatine. Owing to the specific association of the formation of creatinine with the integrity of muscle processes (110, 191), the urinary concentration of this material may give a more reliable indication of the severity of the disease than the level of urinary creatine (103).

In the normal subject there is a rather remarkable relationship between the level of urinary creatinine and the total mass of the musculature. Estimation of the muscle mass on the basis of the creatinine excretion

appears to yield important information regarding the extent to which the muscular system is involved in patients with this disease. This value has been found to vary from 35–40% in incipient progressive muscular dystrophy to values as small as 17% in patients in whom the disease is advanced (103). There was, moreover, a marked correlation between the muscle mass calculated as per cent of body weight and the degree of physical performance of patients with dystrophy, in so far as such performance could be appraised in a quantitative fashion.

Following the elucidation of the role of methionine in the synthesis of creatine and creatinine (222), and the discovery that an exhaustion of methyl groups may occur in the presence of excessive concentrations of methyl acceptors (82, 221), speculation naturally arose over the possibility that exhaustion of methyl stores might arise in progressive muscular dystrophy owing to the phenomenon of excessive creatinuria. Results of studies on the total methyl output in patients with this disease reveal that the loss in methyl groups occurring as a result of excessive creatine output was fully compensated for by a diminution in output of creatinine. There was, moreover, a surprising agreement between the total quantity of "methyl" excreted by the group of normal subjects and that excreted by patients with progressive muscular dystrophy (103). These data suggest that there is no absolute increase in the output of total creatine compounds in progressive muscular dystrophy and that the difference between normal subjects and patients with muscular dystrophy, with respect to the excretion of creatine compounds, resides in a differential partition of these substances in the urine. In view of the fact that creatinine has been shown by means of isotopes to be derived from creatine (18), it would appear that in progressive muscular dystrophy the excessive urinary excretion of creatine does not arise as a result of an increase in the synthesis of creatine, but as a result of the incomplete metabolism of this compound in muscle, with a consequent decrease in the amount converted to creatinine.

Original claims made for the efficacy of glycine and other creatine precursors in effecting improvement in patients with progressive muscular dystrophy (12, 44, 148, 218) have been largely abandoned (26, 111, 144, 165). Most investigators of this disease have come to the conclusion that the abnormality in creatine excretion is a secondary effect arising from an inherent defect in muscle, and that the mechanism for the synthesis of creatine is probably intact (182).

Claims of Bicknell (15), and others (205, 206), that "cases of muscular dystrophy slowly improve or recover when treated with vitamin E" have not been supported by the results of other workers (4, 65, 83, 211). Re-



ports of success in the treatment of progressive muscular dystrophy with pyridoxine (7) have likewise been seriously challenged (49, 61, 65, 131). Doubtless many of the conflicting statements with respect to the efficacy of various agents on the clinical course of progressive muscular dystrophy would be eliminated or resolved if adequate methods were available for the quantitative evaluation of the clinical status of the patient. From a consideration of the known physiology of creatine and creatinine, it is clear that any criterion of improvement in muscle function in this disease would have to include a definite increase in the excretion of creatinine. In none of the reported instances of improvement in progressive muscular dystrophy, following the administration of glycine, vitamin E, or pyridoxine, has convincing evidence of an increase in creatinine excretion been obtained.

Abnormality in carbohydrate metabolism in patients with progressive muscular dystrophy has been described by several investigators. McCrudden and Sargent (133), Byard (30), and Brock and Kay (29) have reported low, fasting levels of blood sugar. Others have reported delayed utilization of dextrose (112, 162). McCrudden (132) expressed the opinion that an abnormality in glucose metabolism was an underlying factor in the malfunctioning of the muscles in dystrophy. Results of later studies, however, have failed to confirm these findings, and reveal normal responses to test doses of glucose given by oral (54), or intravenous routes (194). Studies of the lactic acid levels in the blood of patients with dystrophy have yielded results which are equally conflicting, ranging all the way from levels considerably higher than normal concentrations (162), to normal (172), and lower than normal (189).

Meldolesi (142), in a study of 48 cases of progressive muscular dystrophy, reported decreased urinary excretion of nitrogen, a delay in the secretion of lipase, diastase, and trypsin from the pancreas. From these observations he concluded that the disease was the result of pancreatic dysfunction, with the chief defect an inadequate absorption of nitrogen and a depletion of protein reserves. Results of recent studies of the nitrogen, phosphorus, calcium, and magnesium metabolism in patients with progressive muscular dystrophy fail to demonstrate any significant abnormality (149, 194). Moreover, others have failed to confirm the claim that subjects with dystrophy show significant diminution in the secretory activity of the pancreas (10, 190).

The effect of progressive muscular dystrophy on the chemical composition of muscle has not been investigated extensively. Results obtained on biopsies of dystrophic muscles by Collazo *et al.* (41) were reported as

revealing low contents of inorganic phosphorus, creatine phosphate, glycogen, and water, which were lowered in proportion to the severity of the disease. The figures for lactic acid (188), on the other hand, were approximately normal, as were the percentages of creatine phosphate and total acid-soluble phosphorus. The results were interpreted as indicating that in progressive muscular dystrophy there is depression of the synthesis of creatine phosphate. Reinhold and Kingsley (180) found that the chemical composition of muscle in progressive muscular dystrophy was altered more extensively than in diseases with secondary atrophy of the muscles. The concentration of creatine and other substances extractable by dilute acid were diminished. Creatine phosphate and adenosine triphosphate constituted a smaller proportion, and soluble ester and inorganic phosphorus a larger proportion of the acid-soluble phosphorus when compared with control specimens of human muscle.

Debré *et al.* (46) have reported considerable diminution of total and soluble phosphorus in biopsies of the muscle in three subjects with progressive muscular dystrophy. Percentage of creatine phosphate of total acid-soluble phosphorus was approximately normal in two cases, but lowered in the third. The percentage of adenosine triphosphate was lowered in all three. They did not apparently consider the lowering of the total acid-soluble phosphorus as due altogether to the increase of fat and connective tissue in the muscle. Studies of the respiratory quotient of specimens of muscle removed from patients exhibiting various stages of progression did not reveal values differing significantly from those obtained from specimens of normal human muscle (193). The  $Q_{O_2}$  and  $Q_{CO_2}$  were extremely low in the diseased tissue, but the ratio of carbon dioxide production to oxygen consumption was normal. Nevin (167), who had previously reported a lowered concentration of creatine phosphate, with a normal ratio to the concentration of total acid-soluble phosphorus, questioned the significance of results showing diminution in concentration of muscle constituents in progressive muscular dystrophy, and was inclined to attribute these changes to an increase in fat and connective tissue.

In general, it seems that results of analyses of muscle in progressive muscular dystrophy which have been obtained thus far have had little significance owing to the fact that no suitable basis of reference was available which permitted a comparison of the mass of muscle cells in one specimen with the mass of surviving cells in another. Two specimens of muscle removed at biopsy from approximately the same area, in a subject showing incipient, progressive muscular dystrophy, may show differences of 50% in fat content, 20% in concentration of collagen and fibrous connective

tissue, and 15% in the content of free water and ash (105). Low values for organic and inorganic constituents in muscle showing marked degenerative changes are therefore meaningless, unless some factors of correction can be employed which will correct for the differential mass of muscle cells present in the samples. Some success has been achieved recently in the use of myosin as a base of reference for certain organic constituents of diseased muscle (105). Since myosin is the principal protein component of the contractile cells of muscle, its concentration should bear an important quantitative relationship to the total mass of contractile cells in the specimen taken for analysis. Owing to its critical range of solubility in potassium chloride, myosin can be determined quantitatively on specimens of muscle weighing no more than 100–150 mg. Myosin isolated from the diseased muscle did not differ in physical properties or in adenosine triphosphatase activity from that obtained from normal human muscle (105).

The problem of an adequate base of reference for constituents of diseased muscle is one of the principal barriers to the proper interpretation of analytical results secured on muscle in the muscular syndromes. Practical methods for the determination of residual mass of contractile cells will have to be devised, therefore, before full consideration can be given to data on changes in the concentration of enzymes, substrates, minerals, and metabolites occurring in muscle as a result of disease.

It is evident from the content of this review that most studies on the primary diseases of muscle have been planned on the assumption that the seat of the disturbances in the various syndromes resides in the muscle. In an attempt to elucidate possible common mechanisms responsible for alteration in function and the biological sequence which is observed clinically to accompany the muscular disorders, attention has been centered chiefly on a consideration of the chemical and physiological changes known to be associated with the phenomena of muscular contraction. In most cases, however, no adequate evidence has been obtained which points conclusively to muscle as the site of the initial events which lead to the production of muscle disease. In some cases, it is quite possible that alterations in muscular function observed in these syndromes may arise as a result of inherent defects in other organs or organ systems. Instances of this sort, in which relatively mild pathological changes in one organ are reflected in marked functional and pathological changes in remote organs, are fairly common. It seems, therefore, that future investigations on the muscular diseases should no longer be centered principally on the local changes associated with muscle dysfunction, but should include studies of the most diverse variety on possible aberrant mechanisms in other organs,

in the hope of locating pathological processes responsible for the initiation of chains of events which are recognized clinically as the muscular disorders.

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# ACETYL PHOSPHATE

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## I. Introduction

A differentiation between catabolism and anabolism, tried by an earlier generation of biochemists, is disintegrating with better understanding of the fine chemistry of metabolic processes. Not only are breakdown fragments regularly reencountered as building blocks in synthesis, but even quite complex reaction sequences are recognized to function reversibly, either catabolically as a source of energy or anabolically by absorbing pooled energy to build necessary compounds. We are presented with an intricate metabolic maze which, however, becomes increasingly rationalized with recognition of an interchangeability of metabolic energy units. Anabolism, it seems, will resolve largely into a fitting of chemical energy units into the proper places to effect a linking or relinking of catabolic fragments.

In a previous article (37) the concept of the 12 kg.-cal. phosphate bond ( $\sim$ ph) as a circulating energy unit was developed. The stimulus for suggesting a generality of this metabolic unit derived largely from our ex-

perience in the analysis of pyruvate catabolism, particularly in bacterial enzyme systems (35, 39). It was observed that the initial step in this process of eventual energy liberation was the attraction of inorganic phosphate into the sphere of enzymic action. Subsequent degradation of pyruvate yielded acetyl phosphate with mobilized energy still covered in the energy-rich acyl phosphate bond. It was envisaged that this intermediary represented a metabolic crossroad which might lead into various pathways of synthesis.

During the last few years, the acetyl problem has indeed assumed unexpected proportions. Ingested acetate, when followed with the isotope technique, appears in a great variety of compounds. The fatty-acid chains seem to be synthesized completely by a lining up of acetyl residues (63). Likewise, the ring structure of cholesterol (6) and other sterols (5) is derived partly or wholly by acetyl condensation; and most recently acetic acid was recognized as a building stone of the hemin structure (7). Some of the acetate is also synthesized into liver glycogen by way of the tricarboxylic acid cycle (48).

A functioning of acetyl phosphate in anabolic reactions has since been found in bacterial systems. In animal tissues the role of acetyl phosphate is not yet as well circumscribed (10, 30, 45, 77). Promising progress has been made lately with the finding of the utilizability of phosphate bond energy for enzymic acetylations (40, 53).

The differences in behavior of acetyl phosphate in bacterial systems and animal preparations make it advantageous to deal separately with the results obtained in the two fields. Experiments with bacterial enzymes have been pacemaking and logically will precede, in our discussion, those which more recently have been carried out with animal tissues.\*

## II. Bacterial Metabolism

We like to distinguish between *catabolic* and *anabolic* synthesis of acetyl phosphate. Such a distinction represents to some extent an idealization, a division of the over-all effect into partial and frequently inseparable units. But the impression which the seesaw chemistry of isolated intermediary metabolism seems to give is misleading. The functioning of this chemistry in the intact organism requires organization through a balancing of the flow of energy through chemical channels.

*Catabolically*, acetyl phosphate is formed from inorganic phosphate

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\* Work on the problem in this laboratory was supported by the Commonwealth Fund.

through a coupling of degradation and condensation whereby part of the metabolite's energy is shifted into the energy-rich and easily detachable acyl~phosphate bond. Eventually the energy is delivered into the adenylypyrophosphate pool for distribution.

The *anabolic* process starts with a reversal of the last step of the catabolic process: an acetate molecule derived from a variety of reactions is activated by reaction with adenylypyrophosphate. The acetyl phosphate may continue into condensations, with conversion of the C~ph into C—C (42, 71), C—N (40) or C—O linkages (41a, 55).

### 1. Catabolic Synthesis

#### A. SURVEY

The observation that pyruvate oxidation in cell-free preparations of *Lactobacillus delbrueckii* (*acidificans longissimum*) depended on the presence of inorganic phosphate eventually led to isolation of acetyl phosphate as the C<sub>2</sub> residue in this oxidative decarboxylation (39). The over-all reaction is therefore formulated as:



It is unnecessary to recapitulate in detail the experimental evidence for this equation. Only a group of pertinent data is selected here as illustration. Acetyl phosphate was isolated in pure form from the reaction medium as a crystalline silver salt (39). The stoichiometry of the reaction was verified by two independent methods of assay (39, 44) and recently was duplicated in another laboratory (69). The practical equivalence of oxygen consumed and acetyl phosphate formed is shown in Table I. The theoretical ratio of O<sub>2</sub> to acetyl phosphate equals one, hydrogen peroxide being formed in the enzymic process.

TABLE I

ACETYL PHOSPHATE FORMATION IN EXTRACT OF *Lactobacillus delbrueckii* (44)\*

Incubation time, min.	—O <sub>2</sub> , micromoles	Acetyl phosphate, micromoles	<u>Acetyl phosphate</u> O <sub>2</sub>
30	11.2	10.6	0.95
60	20.1	19.1	0.95

\* 1 ml. of bacterial extract, containing 0.15 M phosphate and 0.02 M fluoride, was shaken with air at 36° in the Warburg apparatus. 100 micromoles of pyruvate was added at zero time. Acetyl phosphate was determined by the hydroxylamine method (44).

The sequence of observations leading to isolation of acetyl phosphate is rather typical and may be summed up in a scheme of tests having a more

general applicability. With only slight modification, the same scheme was successfully applied in the analysis of the various reactions where acetyl phosphate was identified as an intermediary (23, 25, 72).

1. The existence of a phosphorylated link was first indicated by the observation of the *indispensability of inorganic phosphate* in the over-all reaction, be it oxidation or otherwise. If proportionality is found between phosphate concentration and rate of reaction, and the phosphate concentration at saturation is relatively low, then a fixation of phosphate is suggested.

2. A change in the phosphate balance, however, may not be detected with ordinary methods, possibly because of chemical or enzymic *instability of the compound*. Therefore, a trapping of the intermediary may be attempted, either chemically or by coupling the reaction with phosphorylation of an eager phosphate acceptor, for instance, adenylic acid.

3. If, with addition of adenylic acid the test reaction causes transformation of inorganic phosphate to adenylyl pyrophosphate, the suspected existence of an intermediary is practically proved. *Formation of adenylyl pyrophosphate* furthermore places the intermediary phosphate compound in the energy-rich series. Instead of using adenylic acid as the primary acceptor, it will frequently be more practical to employ a secondary acceptor like glucose or others. A phosphate transfer to glucose, occurring to our knowledge by intermediation of adenylic acid, indicates, likewise, an energy-rich bond in the intermediary.

4. When evidence for the existence of an intermediary has been forthcoming, but its chemical identity is still unknown, it appears advantageous to test, as a first check, suspected compounds for a functioning as phosphate donors to adenylic acid or to glucose. By identification of a suspected compound as a phosphate donor, detection of its catabolic formation and eventual identification will be greatly facilitated.

#### B. CHEMISTRY OF ACETYL PHOSPHATE

In the course of our analysis, it became necessary to map the properties of acyl phosphates (42), a group of compounds which had been rather poorly characterized so far. Some data had been given in the literature (49), but the urgent need for an analytical method, in particular, made a more detailed survey of the limitations for the stability desirable. Belonging to the group of acid anhydrides, acetyl phosphate is more or less easily hydrolyzed by water even at room temperature. A mapping of stability related to pH, as in Figure 1, shows minimum decomposition in the slightly

acid region. Of particular analytical interest is the enormous acceleration of the hydrolytic split with molybdate. Largely because of this molybdate effect, the compound had been overlooked previously. As is shown in Figure 2 (see page 236), an acid molybdate solution, as used in the Fiske and Subbarow method, decomposes acetyl phosphate almost instantaneously. In mineral acid alone, the compound deteriorates considerably more slowly.

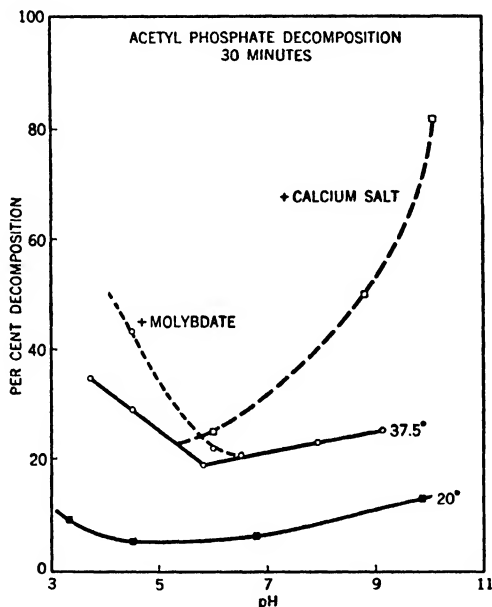
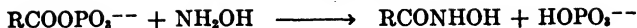


Fig. 1.—Dependence of acetyl phosphate decomposition on *pH* (42).

Two analytical methods have been worked out. The first makes use of the solubility of calcium acetyl phosphate in moderately alcoholic solution (42). True inorganic phosphate may be precipitated with alcoholic calcium chloride solution at *pH* 8, where decomposition is quite slow, 25% alcohol being needed to complete precipitation of calcium phosphate at this low *pH*. The difference of "direct," Fiske and Subbarow phosphate, minus true inorganic phosphate is the acetyl phosphate.

Recently, a more specific and easier method was worked out (44) by making use of a rapid, and under controlled conditions rather specific, reaction between acyl phosphate and hydroxylamine:



The hydroxamic acid formed gives, with ferric ion, a strongly purplish complex which is suitable for colorimetric determination. Any acyl phosphate may be determined by this method. It has also been used for an easy demonstration of phosphoglyceryl phosphate. By adding before

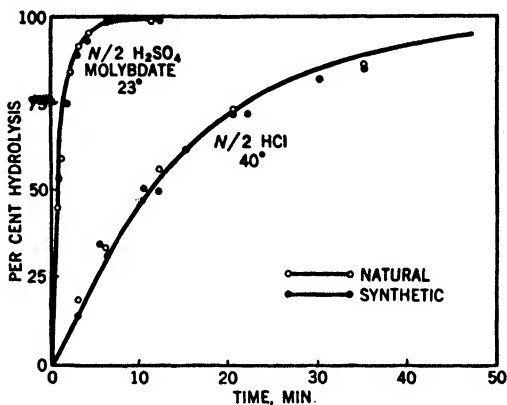
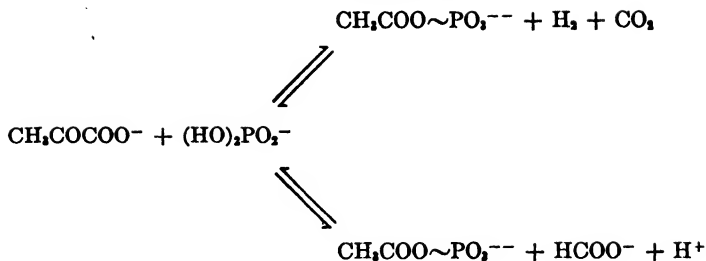


Fig. 2.—Effect of molybdate on the hydrolysis of acetyl phosphate in mineral acid (42).

incubation, hydroxylamine was used successfully as a trapping reagent for acetyl phosphate (45).

### C. THE PHOSPHOROCLASTIC REACTION

The enzymic fission of pyruvate, either to acetate, hydrogen and carbon dioxide, as in *Clostridia*, or to acetate and formate, as in *Escherichia coli*, had been described as a hydroclastic reaction. Johnson and Koepsell (25) with *Cl. butylicum* extracts, and Kalnitsky and Werkman (23) with extracts of *E. coli*, observed independently that the so-called hydroclastic reactions, when studied in isolated enzyme systems, depend upon the presence of inorganic phosphate. Aware of the analogy to pyruvate dehydrogenation in *Lactobacillus delbrueckii*, they eventually identified acetyl phosphate as the primary product of the reaction in both cases (25, 72). Therewith, the so-called hydroclastic reaction becomes, in fact, a phosphoroclastic reaction:



These enzymic fissions of pyruvate are remarkable because they cast new light on the chemistry of the pyruvate molecule and the  $\alpha$ -keto acid grouping in general. Hydrolysis between the keto and carboxyl groups occurs with evolution of considerable energy,  $\Delta F_0$  ca.  $-15$  kg.-cal. A phosphorolysis, however, leading to carboxyl phosphate instead of free carboxyl, saves about 12 kg.-cal. in the energy-rich phosphate bond. The reaction thus becomes practically reversible and anabolically the reverse reaction is capable of functioning as a device to effect a C—C linkage between a carboxylic acid and carbon dioxide, provided hydrogen and energy-rich phosphate bonds are available. This mechanism, as will be explained in more detail subsequently, furnishes a viable enzymic pathway for a reduction of carbon dioxide to the carbohydrate level.

**Acetyl Phosphate from Pyruvate in *Clostridium butylicum* Extracts.**—Active extracts were prepared by prolonged freezing followed by extraction

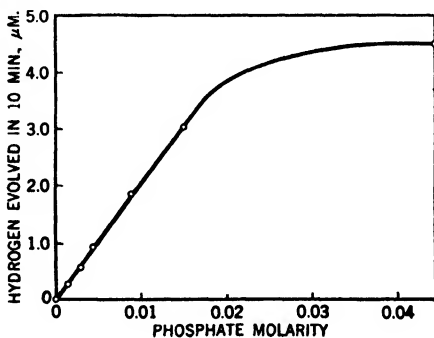


Fig. 3.—Effect of added phosphate on rate of hydrogen evolution (25).

(25). The  $Q_{H_2}$  for pyruvate in such extracts was about 60. Just one mole each of acetic acid, hydrogen, and carbon dioxide were formed per mole of



pyruvate metabolized. Inorganic phosphate was found to be an indispensable component of this system (Fig. 3, page 237).

The addition of glucose more than doubled the reaction rate, and, with glucose stable phosphate esters were formed in amounts equivalent to pyruvate decomposition. In absence of a phosphate acceptor, the formation of a labile phosphate ester was demonstrated by the first method for acetyl phosphate determination of Lipmann and Tuttle (42). With excess phosphate added, amounts of labile phosphate appeared which were approximately equivalent to pyruvate utilization. A further characterization of the labile ester was obtained by isolation of the silver fraction of the organic phosphate and, even without a complete purification, these data practically prove the identity with acetyl phosphate. As expected, these extracts readily transfer phosphate from added acetyl phosphate to glucose, and thus the coupling between glucose phosphorylation and pyruvate degradation is explained.

**Butyryl Phosphate.**—A closer examination of the silver fractions containing labile phosphate revealed the presence of some butyric acid besides acetic acid (26). Therefore, a shift of phosphate from acetyl phosphate to butyric acid was indicated; on incubation of a mixture of synthetic or "natural" acetyl phosphate with butyrate one-third of acetyl-bound phosphorus was transferred to butyric acid. This transfer is most likely mediated by adenylyl pyrophosphate which reacts with butyrate as well as with acetate to form the corresponding phosphoryl derivatives (39). A shift of phosphate from one fatty acid to another is a noteworthy reaction and may be related to Lehninger's recent (31) suggestion of initial phosphorylation of the carboxyl group in fatty acid oxidation.

**Cleavage of Pyruvate to Acetyl Phosphate and Formate (*E. coli*).**—Extracts of *E. coli* which vigorously fermented pyruvate to acetate and formate were prepared by Kalnitsky and Werkman (23) using the glass-powder crushing procedure. The absence of hydrogenlyase makes formate in these extracts a stable end product. The first observation leading to recognition of the phosphoroclastic nature of the reaction was again the indispensability of inorganic phosphate. Extracts inactivated by dialysis could be reactivated with addition of phosphate, saturation being obtained between a molarity of 0.02 and 0.05. After longer dialysis, phosphate, co-carboxylase, and  $Mg^{++}$  or  $Mn^{++}$  were needed for reactivation (Table II). Therefore the phosphoroclastic reaction as well as its reverse is catalyzed by a thiaminpyrophosphate enzyme. The thiamin catalysis of a reversible reaction is noteworthy; it shows that *thiamin enzymes can catalyze a synthesis as well as breakdown of  $\alpha$ -keto acids.*

Utter and Werkman (72) subsequently studied the effect of phosphate more closely and demonstrated that a labile phosphate compound with the properties of acetyl phosphate was the primary reaction product. They

TABLE II

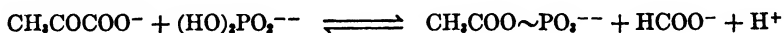
EFFECT OF COCARBOXYLASE, MANGANESE, AND MAGNESIUM ON PYRUVATE DISSIMILATION BY DIALYZED *E. coli* JUICE (23)

Contents of cup*	1 hr.	2 hrs.	3 hrs.
Dialyzed juice + cocarboxylase, $\text{PO}_4^{---}$ , $\text{Mn}^{++}$	645	1123	1281
Dialyzed juice + cocarboxylase, $\text{PO}_4^{---}$ , $\text{Mg}^{++}$	37	325	1054
Dialyzed juice	-37	40	160

\* Cups contained dialyzed juice, 1.0 ml.; pyruvate (0.045 *M*);  $\text{NaHCO}_3$  (0.045 *M*); phosphate buffer (0.02 *M*; *pH*, 6.88); cocarboxylase, 15  $\mu\text{g}$ .;  $\text{Mn}$  or  $\text{Mg}$  (0.005 *M*). Total volume, 2.3 ml. Atmosphere, 10% carbon dioxide in hydrogen.

found that the extent of acetyl phosphate accumulation depended greatly on the concentration of inorganic phosphate. With 0.1 *M* phosphate the ratio, acetyl phosphate/—pyruvate, reached 0.85, approaching closely the theoretical value of one. Acetyl phosphate when added to these extracts is decomposed appreciably, more by enzyme action than by spontaneous breakdown, and inorganic phosphate inhibits strongly the enzymic breakdown.

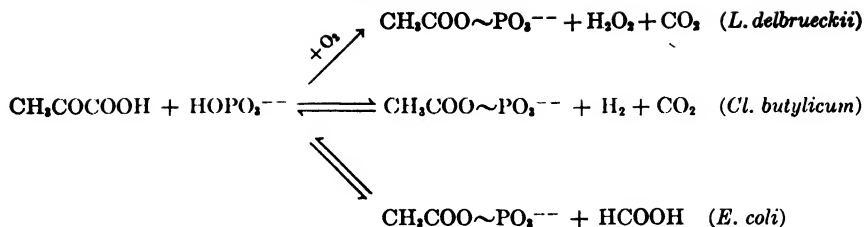
In these extracts, synthetic or natural acetyl phosphate readily yielded phosphate to adenylic acid or to glucose through transphosphorylation. These data led to the formulation of this reaction also as a phosphoroclastic split of pyruvate:



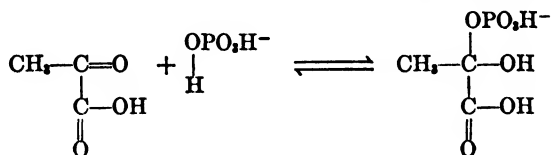
Another organism may be mentioned here which is equipped with transferases connecting acetyl phosphate with phosphate acceptors. Utter, Krampitz, and Werkman (70) found with *Micrococcus lysodeicticus* transphosphorylation to glucose. They used lysed preparations for these experiments and observed there an interesting, apparently specific oxidation of acetyl phosphate which seems coupled with the phosphate transfer.

#### D. CHEMISTRY OF THE COUPLING REACTION

The three bacterial fermentations of pyruvate which lead to synthesis of acetyl phosphate are compared in the following equation:



The common feature in these reactions is a condensation of the acetyl portion of pyruvic acid with phosphate. In the final breakdown phase only, the various enzyme systems deviate into different channels, all passing through an analogous initial stage. Presumably this initiating step is a pairing of pyruvate and phosphate molecules to form, by addition of phosphate on the carbonyl double bond, a semiacetal-like compound:



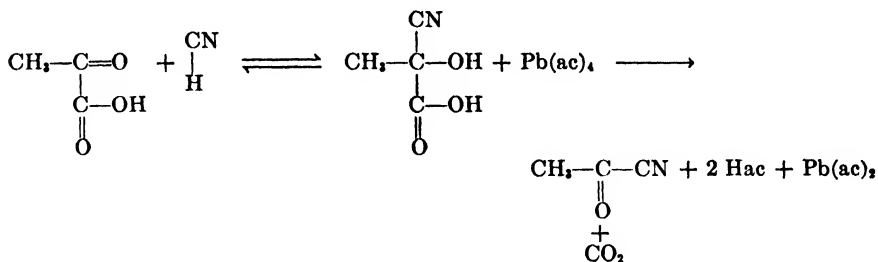
The ease with which the carbonyl double bond is saturated through addition of weak acids, like acid sulfite or hydrocyanic acid, supports this view. It is quite probable, however, that in the case of phosphate the equilibrium is strongly in favor of dissociation. Unpublished experiments show that, even with great excess of phosphate, the strong ultraviolet absorption of the carbonyl in pyruvate,  $\epsilon = 13$  at  $320 \text{ m}\mu$  (20), remains unchanged, although it disappears completely with a slight excess of sulfite. These measurements were done in plain aqueous solutions. In studies, using enzyme solutions, Meyerhof and co-workers (51) obtained negative or inconclusive results for an analogous addition between phosphoglyceraldehyde and phosphate. It seems questionable that an accumulation of the addition product is to be expected outside the enzyme parameter.

In their book on theoretical organic chemistry Branch and Calvin (8), in a discussion of the carbonyl double bond, remark:

"The product of an addition to a carbonyl compound is often a substance that would undergo the reverse reaction quite rapidly. The reactivity of these compounds is therefore a rate rather than an equilibrium phenomenon. In fact in many cases the addition product is an intermediate in a reaction and is not actually isolated."

Such an attitude seems all the more justified in the present case since recently a chemical analogue to our enzymic coupling of cleavage and condensation was discovered by Baer (1) in studies on the action of lead

tetra-acetate on  $\alpha$ -keto acids. To enter into this reaction the keto acid must combine with a compound able to add to the carbonyl double bond as a complement, for instance, with hydrocyanic acid or ethanol. In an inert medium, no reaction occurs without this complement, in the presence of which, however, the ensuing oxidative decarboxylation is coupled with condensation of the complement with the acyl portion:



The above formulation of this reaction, with pyruvate and hydrocyanic acid as reactants, has been pointed out by Baer (2) as showing close analogy to enzymic condensation of acetyl phosphate. With alcohols, the esters of the next lower fatty acid were obtained, and in his latest publication (2) it is mentioned that phosphoric acid may also act as complement in this system. The cleavage mechanism is compared with cleavage between two adjacent hydroxyl-carrying carbons and the function of the complement is to furnish, as a "hydroxyl former," the hydrogen which converts the  $\alpha$ -keto into a pseudohydroxy acid. With water present, this always reacts preferentially and free acid is formed instead of the acid derivative.

Apparently the enzymic system is constructed in such a manner as to allow reaction only with phosphoric acid as complement to the carbonyl. Through this manipulation the cleavage energy is stored in the acid anhydride bond, in an easily detachable position. The biological function of the phosphate may be thought to be in guarding energy carry-over. The insertion of phosphate, instead of water, levels the energy gradient from steep decline to practical reversibility. Thermodynamically, the phosphate mechanism represents a restraint rather than a promotion of the reaction. Therefore, it seems questionable that the phosphate should be considered here as a catalyst. The thermodynamic potential certainly is steeper without the condensation and it is therefore not surprising, perhaps, that some organisms manipulate pyruvate degradation the simpler way, without phosphorylation (69).

2. *Anabolism of Acetyl Phosphate*

In anabolic absorption, the phosphate bond unit enters the reaction system through enzymic transfer from adenyly pyrophosphate. So far the rule seems to hold that transfer on the energy-rich level, or down therefrom, is mediated by the adenylic acid system. A central steering of the flow of bond energy through the adenylic-acid pool may be an important factor in cellular economy although we should be aware that a multiplicity of carrier-enzyme systems, as in hydrogen transport, is not yet excluded.

With anabolic pathways radiating thus from the central adenylic acid system we describe the  $\sim\text{ph}$  transfer from adenyly pyrophosphate to acetate as anabolic synthesis of acetyl phosphate. The readiness with which the reverse reaction, from acetyl $\sim\text{ph}$  to adenylic acid (see Table III), takes

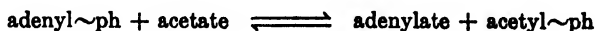
TABLE III  
TRANSFER OF ACETYL-BOUND PHOSPHORUS TO ADENYLYC ACID (39)\*

Incubation time	Adenylic acid added	P <sub>7</sub>	Per cent of $\sim\text{ph}$	P <sub>acetyl</sub>	Per cent of $\sim\text{ph}$	P <sub>inorganic</sub>
			(P <sub>7</sub> + P <sub>acetyl</sub> )		(P <sub>7</sub> + P <sub>acetyl</sub> )	
At start	..	2.1	12	15.0	88	4.0
After 60 min.	15.6	8.0	73	2.9	27	10.0

\* Suspension of 45 mg. of dry *L. delbrueckii* in 1.0 ml. of 0.025 M sodium fluoride solution incubated at 37°. All results are expressed in micromoles. P<sub>7</sub> = P liberated on 7-min. hydrolysis in N hydrochloric acid.

place suggested early an equilibrium quite in disfavor of the donor. Approach to equilibrium from the acetyl phosphate side shifted about 80% of the  $\sim\text{ph}$  to adenylypyrophosphate. Data for equilibrium from adenyly pyrophosphate toward acetate (Fig. 4) indicate a distribution of  $\sim\text{ph}$  even more in favor of adenyly $\sim\text{ph}$ , although with preparations of *L. delbrueckii* as well as with extracts of *Cl. butylicum* appreciable amounts of acetyl phosphate were formed by transphosphorylation. With the *Clostridium* enzyme, butyrate likewise accepts  $\sim\text{ph}$ , and we presume that the observed exchange of  $\sim\text{ph}$  from acetyl $\sim\text{ph}$  to butyric acid (26) is mediated by adenylic acid.

Two sets of data obtained by opposite pairing are graphically rendered in Figure 4 and show the reversibility of the reaction:



A preliminary calculation of a constant from the data yields  $k = 177$  and  $\Delta F_0 + 3.2$  kg.-cal. (39). However, in these experiments a final equilibrium was almost certainly not reached and a lower  $k$  of 50–100 and a  $\Delta F_0$  of  $+2.4$  kg.-cal. are more likely values. The bond energy in this carboxyl $\sim$ ph is therefore approximately 3 kg.-cal. greater than in adenylyl( $\sim$ ph) $_2$ . With a probable 12 kg.-cal. for the ad $\sim$ ph bond the  $\Delta F_0$  for hydrolysis of acetyl phosphate amounts to  $-15$  kg.-cal. This is not too far from the value of  $-12$  kg.-cal. which Meyerhof (50a) recently calculated roughly for the carboxyl phosphate in phosphoglyceryl phosphate. Equilibrium data for the latter compound have not been available to us. Reversibility of the reaction with adenylic acid was mentioned, however, by Warburg and Christian (56, 76) and the enzyme responsible for the transfer was isolated from yeast by Bücher (11).

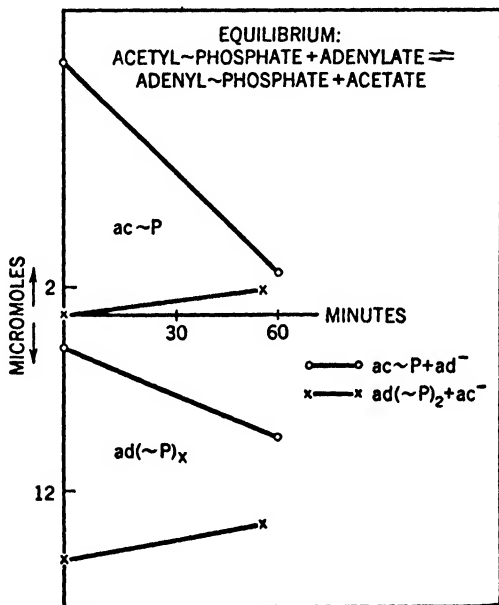


Fig. 4.—Approach to equilibrium of  $\sim$ ph transfer between acetate and adenylic acid.

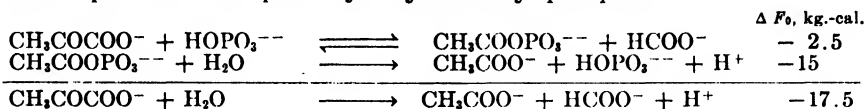
It is of considerable interest that the catabolic unloading of  $\sim$ ph from the metabolite chain to adenylic acid quite generally seems to go downhill while anabolic fitting of  $\sim$ ph into chains has to work uphill and thus requires coordination with compensating reactions. The recently demonstrated  $\sim$ ph transfer between phosphopyruvic and adenylic acid adds another example to the outlined rule. To judge from Lardy's data (29), the shift from adenylyl $\sim$ ph to enol $\sim$ ph seems even steeper. The energy of formation of the enol $\sim$ ph then will be a little higher than of acyl $\sim$ ph, and in

agreement with the *recalculated* value of  $\Delta F_0 = -16$  kg.-cal. for the hydrolysis of phosphoenol pyruvate.

Dr. Crawford F. Failey has called my attention to an error in the earlier reported calculation (37). A recalculation yielding the appreciably higher value, mentioned above, is carried out in detail in an addendum at the end of this article.

#### A. REVERSIBILITY OF THE PHOSPHOROCLASTIC REACTIONS

The role of phosphate in this mechanism appears most clearly when we compare the energy change with *phosphoro*- and *hydroclastic* splits, respectively. The hydroclastic type may be expressed as a sum of phosphoroclastic split and subsequent hydrolysis of acyl phosphate:



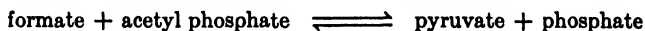
This comparison outlines again the "biotechnological" function of phosphate in this and similar mechanisms: The coupling with phosphorylation covers the bond energy mobilized in the splitting process which then occurs with minimal energy change. With an insertion of water instead, the bond energy is wasted and the pathway back is blocked.

With formic acid and hydrogen plus carbon dioxide being on equal energy levels the same considerations hold in *Cl. butylicum* for the phosphoroclastic split to acetyl phosphate, hydrogen, and carbon dioxide. However, it should be noted that formate is not an intermediary here (25) and that in the reverse reaction carbon dioxide is fixed without passing through a formate stage. This is important for discussions of chemo- and photosynthetic mechanisms.

#### B. THE CONDENSATION OF ACETYL PHOSPHATE AND FORMATE (*E. coli*)

The most complete study of the condensation process was carried out with the enzyme system of *Escherichia coli* where only two metabolites are joined, whereas in *Clostridium* at least three components must be brought together in the synthetic reaction. With the *E. coli* system it was possible to show analytically a recombination to keto acid (43). Approach to equilibrium from both sides is shown in the graph of Figure 5.

From this and similar experiments an approximate value may be obtained for the equilibrium constant of the reaction:



$$k = \frac{\text{pyruvate} \times \text{phosphate}}{\text{acetyl phosphate} \times \text{formate}} = 10^{-3}$$

Through  $\Delta F_0 = -RT \ln k$ , this value leads to about 2.5 kg.-cal. required for the condensation.

**Experiments with  $\text{HC}^{13}\text{OOH}$  (71).**—When heavy carbon containing formic acid was incubated with active *Escherichia coli* extracts together with pyruvic acid and the fermentation was interrupted before all pyruvate had been metabolized, the residual pyruvate was found to contain large amounts of  $\text{C}^{13}$  in the carboxyl group. As shown in Table IV after 60 min. incubation equilibrium was already established between  $\text{HC}^{13}\text{OOH}$  and  $\text{CH}_3\text{COC}^{13}\text{OOH}$ .

The rate of equilibration was found dependent on the phosphate concentration and roughly parallel to accumulation of acetyl phosphate.

The equilibrium between  $\text{C}^{13}$  formate and pyruvate evidences a reverse phosphoroclastic reaction:

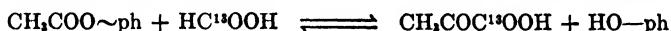


TABLE IV

EFFECT OF PHOSPHATE ON EXCHANGE BETWEEN  $\text{HC}^{13}\text{OOH}$  AND  $\text{CH}_3\text{COCO}^{13}\text{OOH}$  (71)

Expt. No.	Phosphate added, millimole	Acetyl phosphate formed, millimole	Excess $\text{C}^{13}$ , atom per cent		Isotope concentration at start
			Pyruvate $\text{COOH}$	$\text{HCOOH}$	
Nondialyzed					
1	0	0.009	0.59	0.89	$\text{HC}^{13}\text{OOH}$ with 1.72 atom per cent excess $\text{C}^{13}$ added; 45 min. incubation
2	0.5	0.082	0.67	0.75	
Dialyzed					
1	0	...	0.12	1.51	$\text{HC}^{13}\text{OOH}$ with 2.37 atom per cent $\text{C}^{13}$ added; 40 min. incubation
2	0.4	...	0.40	1.14	

**Experiments with  $\text{CH}_3\text{C}^{13}\text{OOH}$ .**—These experiments (71) were particularly instructive as regards the role of  $\sim\text{ph}$  in keto acid synthesis.

TABLE V

EFFECT OF ADENYL PYRROPHOSPHATE ON FIXATION OF  $\text{CH}_3\text{C}^{13}\text{OOH}$  IN PYRUVIC ACID (71)

Adenyl pyrophosphate added, mg. per ml.....	0	0	0.45	0.62
$\text{C}^{13}$ excess in $-\text{CO}-$ of pyruvate.....	0.05	0.02	0.14	0.22

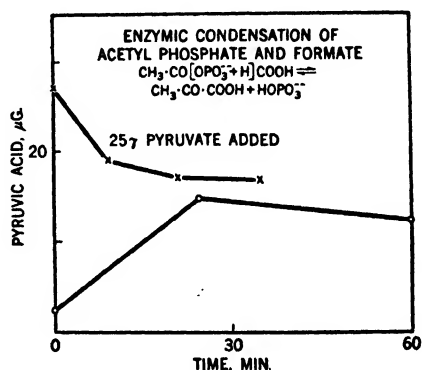


Fig. 5.—Pyruvate formation with acetyl phosphate plus formate (43).

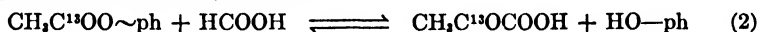


With the heavy acetate and pyruvate alone only doubtful amounts of heavy carbon appeared in pyruvate (see Table V).

The synthesis of keto acid developed first when adenylyl~ph was added together with the heavy acetate. The need of adenylyl~ph shows that phosphorylation of acetic acid is prerequisite to keto acid synthesis. The reaction:

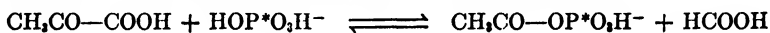


has to precede eventual synthesis:



The relative sluggishness of this sequence, compared with the rapid incorporation of  $\text{HC}^{13}\text{OOH}$ , may be ascribed to the untoward situation of the equilibrium in reaction (1) which causes an availability of only small amounts of  $\text{CH}_3\text{C}^{13}\text{OO} \sim \text{ph}$  to enter reaction (2).

**Tests with Radioactive Phosphate ( $\text{P}^*$ ).**—When the phosphoroclastic split of pyruvate is reversible, a rapid turnover between inorganic and acetyl phosphate is to be expected through the following reaction:



When inorganic  $\text{P}^*$  was added to extracts of *E. coli* together with acetyl phosphate, rapid exchange occurred as expected (43). In 40 min., a turnover of 60% had taken place between the inorganic and acetyl phosphates. This is quite comparable to the formate-pyruvate turnover shown in Table IV. Therefore, the results with heavy carbon and with radioactive phosphate complement each other quite agreeably.

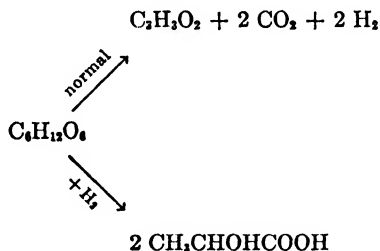
**Fixation of Carbon Dioxide in Pyruvate Carboxyl with Living *E. coli*.**—The cell-free preparations used in the extract experiments did not contain hydrogenlyase (72) but whole cells do, particularly when grown in glucose anaerobically (79). If such cells are incubated with  $\text{NaHC}^{13}\text{O}_3$ , formate, and pyruvate, through the hydrogenlyase formate and bicarbonate become rapidly equilibrated, yielding  $\text{C}^{13}$  formate, and then  $\text{C}^{13}$  begins to appear in the carboxyl of pyruvate (71). Although the Wood and Werkman carbon dioxide fixation via oxalacetate may eventually yield pyruvate with  $\text{C}^{13}$  in the carboxyl, this pathway would contribute none or little under the conditions of above experiments.

#### C. REVERSIBILITY OF THE CLEAVAGE OF PYRUVATE IN *Clostridium butylicum*

It is obvious from the preceding discussion that a split to hydrogen, carbon dioxide, and acetyl phosphate is thermodynamically a reversible re-

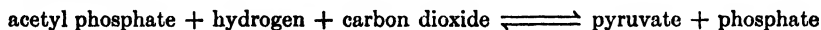
action. The predicted hydrogenative carboxylation was verified experimentally with formate as an intermediary, as shown in the preceding paragraph. In the *Clostridium* extracts, however, formate had been excluded as a link in the reaction. This enzymic mechanism is not merely a summation of hydrogenlyase and the enzyme system, as in *E. coli*, but appears as a homogeneous enzyme catalysis. A condensation of hydrogen plus carbon dioxide plus acetyl phosphate to keto acid through this integrated enzyme system required separate proof. With elimination of the formation of formate as an obligatory step, the applicability of this type of  $\alpha$ -keto acid synthesis as a pathway of reductive carbon dioxide fixation is considerably increased.

**Inhibition by Hydrogen.**—Some time ago, reversibility was suggested by the observation that the butyric acid fermentation in *Cl. butylicum* may be inhibited by hydrogen, *viz.*, one of the fermentation products. Kubowitz (28) found that the production of butyric acid, carbon dioxide, and hydrogen in glucose fermentation by these organisms was inhibited by hydrogen and, to a greater extent, by carbon monoxide, lactic acid being formed instead:



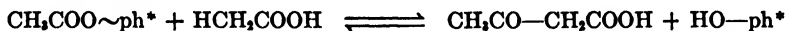
It was assumed that hydrogen intercepted the initially formed pyruvate. More recently an analogous inhibition by hydrogen of the phosphoroclastic enzyme in *Clostridium* extracts has been observed (43).

**Phosphate Exchange.**—As in extracts of *E. coli*, in cell-free preparations of *Cl. butylicum* a rapid exchange may be observed between inorganic and acetyl phosphate (43). Experiments with radioactive phosphate showed that equilibration is brought about even more rapidly; in less than half an hour complete exchange had taken place, in evidence of reversibility of the reaction:



It seems, however, not unlikely that this reaction is not the only pathway for exchange in preparations of this organism. Previous chemical experiments (14) and recent more conclusive isotope experiments (78) have

demonstrated a derivation of the four-carbon chain in butyl fermentations by condensation of two acetic acids. With acetyl phosphate as an intermediary in this fermentation, a mechanism analogous to acetyl phosphate-formate condensation seems likely. Such a reaction could present a pathway for exchange:



Similar questions arise from recent observations on the formation of acetoacetic acid in animal tissues (10, 77), and the probability that acetyl phosphate plays a role in this complex of reactions will be discussed later in detail.

#### D. RELATION TO CHEMO- AND PHOTOSYNTHESSES

**Fixation of Carbon Dioxide in Lactic Acid.**—An enzymic pathway for a chain of reactions leading from  $\text{R}-\text{COOH} + \text{CO}_2$  to  $\text{RCHOH}-\text{COOH}$  appears to be a rather consequential observation. This path is essentially a conversion of carbon dioxide to  $-\text{CH}_2\text{O}-$ , or to carbohydrate level. Slade *et al.* (68) have observed carbon dioxide fixation in lactate as a side reaction with clostridia and suspected  $\text{C}_2-\text{C}_1$  condensation. More recently Brown *et al.* (9) extended these observations. Using a more suitable medium they obtained considerably larger amounts of lactic acid. The following  $\text{C}^{13}$  distribution was obtained: per 100 millimoles of glucose, 55.4 millimoles of lactic acid with 1.89% excess of  $\text{C}^{13}$  in the carboxyl group, and 9.5 millimoles of formic acid with 1.60% excess  $\text{C}^{13}$ . These were the only fermentation products containing  $\text{C}^{13}$  and 87% of the total fixed carbon appeared thus in lactate. The formate contained significantly less  $\text{C}^{13}$  than the lactate, excluding formate as an intermediary in this carbon dioxide fixation. In complementary experiments the possibility of a  $\text{C}_2-\text{C}_1$  condensation was rather rigidly excluded and a reductive carboxylation of acetyl phosphate to pyruvate with subsequent hydrogenation to lactic acid remains the only pathway.

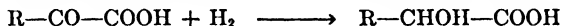
Thermodynamic data for a conversion of carbon dioxide to  $-\text{CHOH}-$  by way of  $\text{R}-\text{COOH} + \text{CO}_2 \rightarrow \text{RCHOH}-\text{COOH}$  are collected in Table VI (43). They indicate that with active hydrogen on the oxidation-reduction level of molecular hydrogen, and with an adequate supply of  $\sim\text{ph}$  these enzyme systems bring about a chemosynthetic reaction liberating 5-6 kg.-cal. per mole carbon dioxide reduced to  $-\text{CHOH}-$ . A detailed survey of these data, in Table VI, reveals that the first step, the reductive  $\alpha$ -carboxylation:



TABLE VI  
THERMODYNAMIC DATA FOR PARTIAL ENZYME REACTIONS OF REDUCTIVE  
CARBOXYLATION (43)

Reaction	$\Delta F_0$ , kg.-cal.	Enzyme system used	Refer- ence
$\text{CH}_3\text{COO}^- + \text{ad}\sim\text{PO}_3^{--} \rightleftharpoons \text{CH}_3\text{COO}\sim\text{PO}_3^{--} + \text{ad}^-$	+ 3.0	<i>Clostridium</i>	39
$\text{CH}_3\text{COO}\sim\text{PO}_3^{--} + \text{H}_2 + \text{HCO}_3^- \rightleftharpoons$ $\text{CH}_3\text{COCOO}^- + \text{HOPO}_3^{--} + \text{H}_2\text{O}$	+ 2.6	<i>E. coli</i>	42, 79
$\text{CH}_3\text{COCOO}^- + \text{H}_2 \rightarrow \text{CH}_3\text{CHOHCOO}^-$	-11.4	<i>Clostridium</i> , <i>Gonococcus</i>	4, 9
Over-all: $\text{CH}_3\text{COO}^- + 2 \text{H}_2 + \text{CO}_2 + \text{ad}\sim\text{PO}_3^{--}$ $\text{CH}_3\text{HOPO}_3^{--} \rightleftharpoons \text{CH}_3\text{CHOHCOO}^- + \text{adH} +$	- 5.8		

is uphill, with an appreciable positive  $\Delta F_0$ , but this is more than compensated for by the strongly exergonic second hydrogenation:

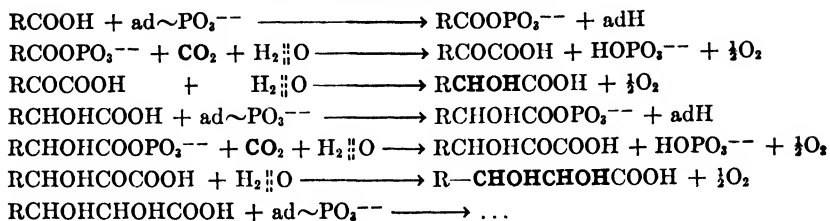


The smoothness of the functioning of such a sequence will depend on the coordination of the consecutive steps. A coupling of compensating partials into an integrated complex reaction is, however, more and more frequently observed in cellular chemistry.

It is not always realized that an energy compensation of similar magnitude is necessary also with  $\beta$ -carboxylation. For instance in the Wood-Werkman reaction, pyruvate + carbon dioxide  $\rightarrow$  oxalacetate, fixation is a rather endergonic reaction,  $\Delta F_0$  ca. + 5 kg.-cal. (16), and appreciable condensation is possible only if coupled with hydrogenation of oxalacetate to malate ( $\Delta F_0 = -11$  kg.-cal.) (16) or if  $\sim\text{ph}$  is available (73). The same is true for Ochoa's carbon dioxide fixation (61) through the series, ketoglutarate + carbon dioxide  $\rightarrow$  oxalosuccinate + 2 H  $\rightarrow$  isocitrate, again a  $\beta$ -carboxylation, to be driven by hydrogenation because spontaneous equilibrium favors dissociation. One may then suspect that the initial carbon dioxide pick-up in chemo- and photosyntheses is not due to a straight equilibrium reaction, grossly favoring carboxylation, but rather to an enzyme complex integrating carbon dioxide fixation and hydrogenation, presumably by way of phosphorylation.

A scheme like that in Table VII (43, 64) may serve for both chemo- and photosyntheses, photosynthesis being more or less a chemosynthesis with water plus light as hydrogen donor (18, 74). The sequence (1) acyl phosphorylation, (2) hydrogenative carboxylation to  $\alpha$ -keto acid, and (3) hydrogenation of the keto to hydroxy acid is repeated infinitely, starting each cycle with phosphorylation of the newly formed carboxyl group. In this manner a polyonic acid of theoretically unlimited chain length may be

TABLE VII  
SCHEME OF PHOTOSYNTHESIS BY ALTERNATING PHOSPHORYLATION  
AND PHOTOREDUCTION (43, 64)



formed. Ruben, Kamen, and Hassid (65) in fact found that the chemical characteristics of the primary product of photosynthesis and chemosynthesis in green plants suggest a polyonic acid of rather high molecular weight.

In this cycle, phosphorylation appears as an initial energy expense, but enough space is in the total energy balance for such a relatively small investment of energy. Ochoa's data on the efficiency of oxidative  $\sim\text{ph}$  generation (58) suggest that oxidation of a pair of substrate hydrogens can yield up to 3  $\sim\text{ph}$ . In the scheme a single  $\sim\text{ph}$  is needed for a complete reduction cycle of carbon dioxide to  $-\text{CHOH}-$ . Therefore, for every three carbon dioxide molecules reduced one extra pair of hydrogens would have to be expended.

Such an initial investment in phosphorylation of carboxyl groups appears to be almost obligatory in *chemosynthesis*; any chemical carbon dioxide fixation must yield primarily a carboxylic acid. But the reduction potential of a free carboxylic acid seems almost too low to be attained in a biological system. The acyl phosphate system, however, with a 0.25 v. more positive potential (37) reaches into the cellular O/R range, approaching closely the region of molecular hydrogen (37). The light reaction theoretically may, however, yield potentials of great negativity. Nevertheless it appears more practical in a water system to avoid potentials far below the hydrogen level. The results of Vogler, LePage, and Umbreit (75) with photosynthesis in sulfur bacteria suggest so strongly a coupling at large with phosphorylation that the above considerations seem to transcend mere speculation.

### III. Animal Tissues

In contrast to the relatively clear picture in bacteria, the extent to which acetyl phosphate participates in animal metabolism is not yet fully understood. The study of its role has been severely impeded by an unexpectedly rapid enzymic destruction of the compound in all tissue preparations (36).

Some enzymic breakdown also had been encountered in bacterial extracts but had been relatively easily overcome by suitable inhibitors. The intensity of enzymic action in tissues, however, was so strong that even in the presence of inhibitors, sufficient activity remained to cause some interference. However, the early observed activation in tissues of pyruvate degradation by inorganic phosphate (3) and its coupling with abundant generation of energy-rich phosphate bonds (12, 57, 58) is difficult to reconcile with a mechanism which is essentially different from that encountered in bacterial systems. Quite recently it has been possible also, through a trapping mechanism, to demonstrate a phosphorylation of acetate, in one instance at least, in pigeon liver (45), although added synthetic acetyl phosphate has so far appeared inactive as a phosphate or acetyl donor (unpublished results). There was observed, furthermore, an over-all dependence of enzymic acetylation on the availability of phosphate bond energy (40, 55), whereby phosphorylation of acetate appears as an integrated rather than as a detachable phase.

### 1. *Acetyl Phosphatase*

Figure 6 illustrates the rapidity of acetyl phosphate breakdown in homogenates of pigeon liver. The enzyme interferes so strongly with attempts to obtain clear-cut answers to questions concerning the metabolism

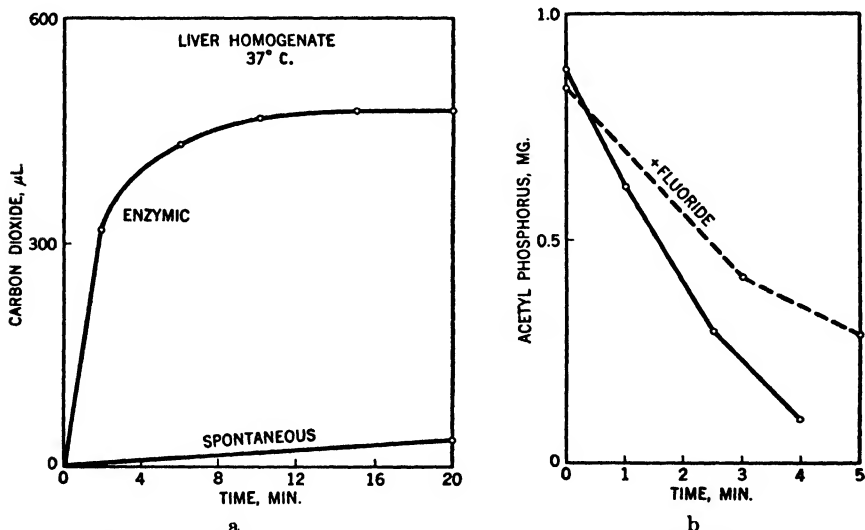


Fig. 6.—Enzymic hydrolysis of acetyl phosphate in 1 ml. of homogenate: (a) manometric; (b) chemical determination (45).

TABLE VIII

ACETYL PHOSPHATASE ACTIVITY IN ANIMAL TISSUES AND BACTERIAL PREPARATIONS\*

Preparation	Units†
Pigeon muscle.....	80
Beef kidney.....	36
<i>E. coli</i> .....	0.7
<i>L. delbrueckii</i> .....	0.1

\* Unpublished results.

† Per gram tissue or per milliliter bacterial juice.

of acetyl phosphate that it became most desirable to acquire a better working knowledge of its action (40, 55).

The enzyme was found to be present in all animal tissues. In order to facilitate comparison, an arbitrary unit was defined and, in Table VIII,

activities in some animal tissues and in bacterial extracts are compared. It appears that kidney is about fifty times and muscle over a hundred times more active than the most active bacterial system. Of the microorganisms studied, *E. coli*, with 0.7 unit per ml., showed the greatest activity, preventing accumulation of acetyl phosphate almost completely in the absence of relatively high phosphate concentrations which inhibit the enzyme (see Fig. 7).

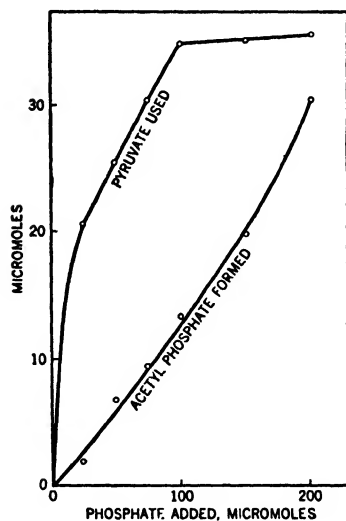


Fig. 7.—Pyruvate dissimulation in *E. coli* extract (72). Dependence of acetyl phosphate accumulation on phosphate concentration.

At higher phosphate concentrations, the effect is due solely to inhibition of hydrolysis at lower concentrations, also to a stimulation of pyruvate breakdown (72).

The acetyl phosphatase of animal tissues is easily brought into solution. It is very stable, not losing activity on storage in the cold; meat bought in the butcher shop and that freshly taken out of the

animal showed comparable activities. The muscle enzyme has the most suitable properties although it is probably not essentially different from enzymes in other tissues. It has been studied most extensively (unpublished results).

The high activity in muscle by itself proves specificity because the common phosphatases are practically absent from muscle preparations. Accordingly tests with our most highly purified muscle enzyme showed no trace of activity with phosphoglycerol. Butyryl, propionyl, and succinyl phosphates, however, were split practically as fast as acetyl phosphate. The assumption of an enzymic nature of the catalysis is based on the observations that (a) the principle is nondialyzable and (b) is completely destroyed by pepsin.

The most interesting feature of the enzyme is its heat stability, particularly at acid reaction. At pH 3-4, prolonged heating in boiling water is practically without effect on the activity of muscle extracts. With kidney preparations the recovery is less complete, although there too, up to 20% may be recovered after heating of acidified extracts. The enzyme, furthermore, is fairly insensitive to trichloroacetic acid. These properties permitted partial purification of the enzyme; and, through heating, trichloroacetic acid precipitation, and acetone fractionation, a concentrated preparation was obtained which contained 18 units per milligram of dry matter. Such preparations showed the properties of a basic protein; they were practically insoluble in water but easily soluble in dilute acetic acid.

The properties just described differ greatly from the known properties of other phosphatases. Whereas, to our knowledge, no heat-stable phosphomonoesterase had been described, it may be recalled that ribonuclease (15), which is presumably a di- or polyphosphoesterase, is a heat-stable enzyme. In muscle, an interesting group of enzymes with analogous properties has been found. Curtius and Ohlmeyer (13) observed the heat resistance of a number of phosphotransferases on the energy-rich level, for instance those linking phosphopyruvate and adenylic acid or creatine and adenylyl pyrophosphate. More recently, a new member of this group was discovered by Kalckar (22), myokinase, a phosphodismutase, which catalyzes the dismutation of two molecules of adenylyl monophosphate to adenylyl diphosphate and adenylic acid. It may be of significance that acetylphosphatase is apparently related to the transferases, although tests for a transfer function, also with purified preparations, have been unsuccessful.

Related to the basic nature of the enzyme protein may be the inhibition by a larger number of polyvalent anions. As mentioned before, inorganic phosphate is a potent inhibitor; similar activity is shown by pyrophosphate and hexose phosphates. Somewhat less strong is inhibition by sulfate, citrate, and oxalate. Fluoride is surprisingly less active, particularly



in purified preparations. Of some special interest is the inhibition by high-molecular anionic compounds, like the nucleic acids and hyaluronic acid.

Shapiro and Wertheimer (67) have independently observed the rapid enzymic breakdown of acetyl phosphate in homogenates and extracts of liver, muscle, kidney, and brain, but did not find such activity in blood serum. That the enzyme is not identical with phosphatase may be concluded from the different distributions. It is believed that the presence of the enzyme may explain negative results with acetyl phosphate as substrate, and a possible role of acetyl phosphate as intermediary is discussed.

## 2. *Coupling between Pyruvate Oxidation and Phosphorylation*

The problem of pyruvate oxidation will not be examined here in its entirety. We shall concern ourselves merely with such results as may contribute to an elucidation of the primary reaction, the oxidative decarboxylation, and shall consider especially the part played by phosphate. Attempts at a mechanical isolation of this reaction have not been very successful. Present evidence suggests a rigid enzymic linkage between dehydrogenation and decarboxylation (34, 59) without an aldehydic intermediary, as in *L. delbrueckii*, in spite of the possibility of breaking down tissue to fractions in which anaerobic, yeastlike decarboxylation appears (19).

A functional separation of the oxidation of pyruvate to acetic acid plus carbon dioxide has been achieved by various means. Anaerobically, the degradation generally stops at the acetic-acid stage, which may be reached either through dismutation (27, 34) or through reduction of methylene blue (34). A further means for separating functionally the primary  $\alpha$ -keto acid dehydrogenation was found by Long and Peters (47) in the use of  $\alpha$ -ketobutyrate in brain dispersion where the breakdown process, even aerobically, ends after oxidative decarboxylation to propionic acid and carbon dioxide, the propionate remaining untouched by the brain. The relationship observed between such isolated oxidative decarboxylation and phosphate turnover is pertinent to the present problem.

A general survey of the ratio of pyruvate oxidation to phosphate concentration appears in Figure 8. The upper curve, computed from data of Banga, Ochoa, and Peters (3), refers to complete oxidation in brain dispersion; the lower curve, adapted from a paper by Long (46), relates phosphate to the rate of anaerobic reduction of methylene blue with pyruvate or ketobutyrate as substrate in washed brain. As shown in Figure 8, the dependence of anaerobic dehydrogenation on the presence of phosphate makes it quite clear that phosphate in some manner participates in this partial reaction.

The problem of phosphate intervention is reflected further by the role of adenylic acid in pyruvic oxidation. A supplement of adenylic acid to dialyzed preparations greatly increases total oxygen consumption and  $\sim$ ph generation per mole of pyruvate (57). Particularly, Long's subsequent experiments make it clear (46) that adenylic acid is mainly or exclusively concerned with the post-acetyl stage; it is not needed anaerobically with pyruvate, nor with ketobutyrate, either for aerobic or anaerobic breakdown. But absence of adenylic acid must stop  $\sim$ ph transfer, and it seems likely then that adenylic acid enhances the completeness of oxidation by

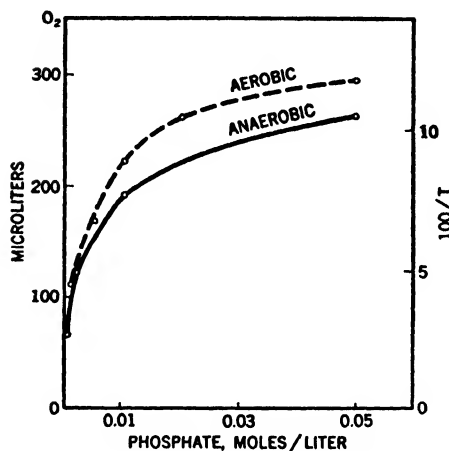


Fig. 8.—Dependence of pyruvate oxidation in brain on phosphate (3, 46).

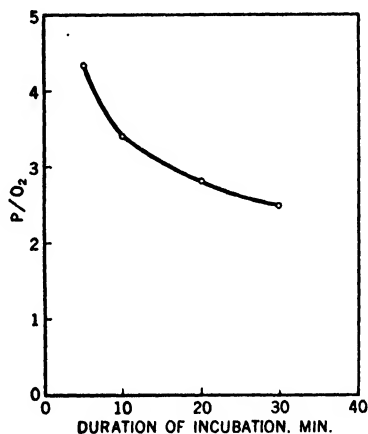


Fig. 9.—Phosphorylation/oxygen consumption (57). Extrapolation to zero time shows that more than 4  $\sim$ ph, per O<sub>2</sub>  $\rightarrow$  2H<sub>2</sub>O, may be generated.

preventing a stagnation through removal of initially formed phosphorylated intermediaries. It is noteworthy that, in the phosphoroclastic reaction in *Cl. butylicum* also, addition of a phosphate acceptor may speed considerably the rate of reaction (26).

Despite its appearance under various conditions as the primary product of pyruvate breakdown, free acetate seems to be a degenerated intermediary. Apparently it cannot be respired by brain under conditions in which pyruvate easily passes beyond the acetate stage (47).

All these observations may then be integrated into the conclusion that phosphate in some manner participates in an oxidative decarboxylation which yields an active C<sub>2</sub> compound. This compound may either degener-

ate to acetate or join immediately in the next step, which is presumably a condensation with a  $C_4$  dicarboxylic acid.

The chemical rationalization of a participation of phosphate in pyruvate dehydrogenation leads almost unavoidably to a scheme analogous to the reaction in *Lactobacillus delbrueckii*, with acetyl phosphate as an enzyme-bound intermediary stage. Theoretically, there still remains a remote possibility that, after its derivation from pyruvate, the electron pair may pass through an independent cycle of  $\sim$ ph generation (38).

It may then be concluded from the foregoing that there is much evidence in favor of a coupling of the immediate process of pyruvate dehydrogenation with phosphorylation although the evidence is not quite conclusive. In this connection it should be recalled that the great abundance of  $\sim$ ph generation with pyruvate oxidation can only be rationalized chemically when a single pair of electrons, on its passage from substrate to oxygen, passes through several  $\sim$ ph cycles. Two to three  $\sim$ ph must be generated with every single transfer,  $RH_2 \rightarrow \frac{1}{2}O_2$  (see Fig. 9), according to Ochoa's observation of ten to fifteen phosphorylations per mole of pyruvate (58). In every complete oxidation of one pyruvate, therefore, ten to fifteen times phosphate is bound as  $\sim$ ph in some manner and unloaded to adenylic acid. This indicates a multiplicity of phosphorylated catalysts and intermediaries, which remain largely unexplained even if acetyl phosphate is assumed to be one of them (38).

### 3. Phosphorylation of Acetate

In competition with the powerful hydrolytic activity of tissue, the reactivity of hydroxylamine with acyl phosphates became a helpful tool in

TABLE IX  
PHOSPHORYLATION OF ACETATE IN PIGEON LIVER EXTRACTS (45)\*

Acetate, millimole.....	10	3	1	..	10
Labile adenylypyrophosphorus, millimole.	10	10	10	10	..
Hydroxamic acid (= ac $\sim$ ph), millimole.	1.76	1.77	1.35	0.90	0.38

\* 0.5 ml. of pigeon liver extract (1 g. of acetone-dried liver extracted with 10 ml. of 0.02 M bicarbonate solution and centrifuged) was incubated for 60 min. at 37° in test tubes. The final volume was 1 ml. All samples contained 0.02 M neutralized hydroxylamine hydrochloride.

trapping acetyl phosphate and saving it from the action of the enzyme. In the presence of hydroxylamine, any acetyl phosphate produced reacts im-

mediately to form acetoxyhydroxamic acid, which subsequently is determined colorimetrically (44). Unfortunately, the reactivity of hydroxylamine with carbonyl groups prevents a utilization of this procedure in the study of pyruvic or acetoacetic acid metabolism.

By use of hydroxylamine as interceptor, a phosphate transfer from adenylyl pyrophosphate to acetate is indicated in pigeon liver extracts (45). The reactivity of acetate—which in  $M/1000$  concentration still reacts appreciably as  $\sim$ ph acceptor—is remarkable (Table IX).

If the concentration of hydroxylamine in liver extracts is considerably increased, a reaction appears between higher fatty acids, from  $C_4$  to  $C_{10}$ , and hydroxylamine (45). This reaction is independent of adenylyl pyrophosphate. From recent unpublished experiments we may attribute to liver lipase this  $\sim$ ph-independent activation of the carbonyl group.

A subtler study of this phosphate transfer has shown it to be catalyzed by an unexpectedly complex system. The transfer from adenylyl $\sim$ ph to acetate is inactivated by autolysis and dialysis (unpublished data); some incomplete reactivation is brought about with addition of boiled liver. More effective are liver fractions rich in a coenzyme for acetylation of sulfanilamide (Table X). The parallelism between these two reactions is remarkable. More details will be given in the next section.

TABLE X

ACTIVATION OF ACETATE PHOSPHORYLATION IN DIALYZED PIGEON LIVER EXTRACT

Glutathione	+	+	+	+
Activator	—	—	+	+
Acetate	+	—	+	—
Adenylyl pyrophosphate	+	+	+	+
Hydroxamic acid (= ac $\sim$ ph), millimole	0.32	0.32	1.24	0.86

So far, tests for analogous reactivity of acetate with the extracts of the livers of other animals or with other organs were unsuccessful. We consider it quite possible that this absence is only apparent and due to partial enzyme destruction.

#### 4. Mechanism of Acetylation

The enzymic acetylation of sulfanilamide in liver was chosen as a test reaction (40). The mechanism of this reaction is now clarified to some extent. Earlier experiments by Klein and Harris (24) with liver slices had shown an energetic coupling of acetylation with respiration. This coupling

indicated a chain of reactions transferring oxidation-derived energy to the energy acceptor, the acetylation system. The isolation of acetyl phosphate made it appear likely that this energy transfer occurred by way of phosphate-bond energy (37). When, in homogenates of pigeon liver, a cell-free preparation became available for study of the finer mechanism (Table XI), the test for a  $\sim$ ph chain as a link was possible (Table XII). It appeared then that adenyl pyrophosphate would replace anaerobically the respiratory reac-

TABLE XI\*  
EFFECT OF METABOLITES ON AEROBIC AND ANAEROBIC SULFANILAMIDE  
CONJUGATION (40)

Substrate added and final concentration	Aerobic				Anaerobic			
	Incubation time, min.	Sulfanilamide, $\gamma$			Incubation time, min.	Sulfanilamide, $\gamma$		
		Direct <i>1</i>	After hydrolysis <i>2</i>	Acetylated <i>2 - 1</i>		Direct <i>1</i>	After hydrolysis <i>2</i>	Acetylated <i>2 - 1</i>
0.....	0	630	630	0	0	442	440	0
0.025 <i>M</i> acetate.....	15	495	640	145	30	420	452	32
0.04 <i>M</i> acetyl phosphate.....	..	370	642	272	..	379	455	76
0.018 <i>M</i> acetoacetate.....	..	...	...	...	..	400	446	46
0.025 <i>M</i> pyruvate.....	..	412	615	203	..	...	...	..
0.013 <i>M</i> diacetyl.....	..	436	650	214	..	387	443	56
0.013 <i>M</i> acetoin.....	..	478	625	147	..	393	437	44
0.013 <i>M</i> acetoin.....	..	474	650	176	..	...	...	..

\* Samples of 1 ml. of homogenate (0.4 g. of liver tissue plus 0.6 ml. of a solution containing 0.7% potassium chloride, 0.01 *M* magnesium sulfate, and 0.03 *M* dipotassium hydrogen phosphate) in a total volume of 1.4 ml. were shaken at 37° in Warburg vessels in an atmosphere of either air or nitrogen. The respiratory activity of the preparation was comparable to that used in the experiments of Table I.

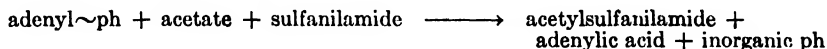
TABLE XII\*  
ANAEROBIC ACETYLATION WITH ADENYL PYROPHOSPHATE (40)

Gas phase	Adenyl pyrophosphate, mg. P <sub>1</sub>	Conjugated sulfanilamide, $\gamma$
O <sub>2</sub> (5% CO <sub>2</sub> )	0	0
N <sub>2</sub> (5% CO <sub>2</sub> )	0.32	55
N <sub>2</sub> (5% CO <sub>2</sub> )	0.16	31

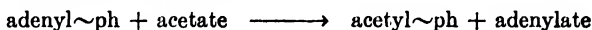
\* At the start, 129  $\gamma$  of sulfanilamide and sodium fluoride, 0.07 *M* final concentration, were added. Incubation time 40 min.

tions on which acetylation depended in the intact cell [see also Nachmansohn and Machado (55)]. Acetyl phosphate, however, appeared inactive (see Table XI), although the rapidity of acetylation in these homogenates made it difficult to ascribe the complete absence of an acetyl phosphate ef-

fect merely to removal by enzymic hydrolysis. On the other hand, the ability of adenylyl pyrophosphate to promote acetylation points rather strongly toward activation of acetate by phosphorylation. These apparently contradictory results suggest an enzymic organization of the over-all process into an impenetrable unit. Such organization would be advantageous because the over-all reaction



is considerably exergonic,  $\Delta F_0 = -9$  kg.-cal. and will easily go to completion. An intermediary step, however:



( $\Delta F_0 = +3$  kg.-cal.) is appreciably endergonic (see page 243). If conducted as a separate enzyme reaction, this step would have to overcome a considerable energy hump, which would be avoided if the reaction were organized on a single center. Such organization would explain why added acetyl phosphate is not active; it cannot penetrate into the fenced-off field of reaction. Nevertheless, as recently observed by N. O. Kaplan in this laboratory, hydroxylamine in concentration as low as 0.001 *M* can inhibit acetylation, presumably through trapping of the activated acetic acid.

The operational advantage of smoothing out the energy hump of carboxyl phosphorylation makes it attractive to picture a general use for adenylyl~ph in cell metabolism as a condensing agent in the manner outlined. As model reactions, esterification of alcoholic hydroxyl in acetylcholine and "peptidation" of an aromatic amine in sulfanilamide acetylation forecast the possibility of application of such a scheme to fat and protein synthesis.

**Properties of the Coenzyme (41).**—A closer examination of the acetylation system in liver has disclosed participation of a heat-stable factor in this reaction. This coenzyme is easily decomposed by an as yet unidentified enzyme present in liver. After prolonged incubation in the cold, with or without dialysis, pigeon liver extracts become inactive but may be reactivated on addition of boiled liver (Table XIII). Experiments with purified liver fractions showed, furthermore, that dialyzed extracts need a sulfhydryl compound for reactivation in addition to the coenzyme. The coenzyme responsible for this activation is rather ubiquitous in animal tissues. Liver, brain, and pigeon breast muscle are especially rich in the substance. Other organs contain less; rabbit muscle contains very little. Boiled extracts of fresh bakers' yeast contained fair amounts; earlier negative results with yeast macerate had led to the erroneous statement of absence of the coenzyme from yeast (41). On fractionation with heavy-

TABLE XIII\*

## REVERSIBLE INACTIVATION THROUGH DIALYSIS OR AUTOLYSIS (40)

Treatment of extract	Filtrate of boiled organ added corresponding to g. fresh wt.	Sulfanilamide conjugated, $\gamma$	Incubation time, min.
Untreated	.....	69	65
Keep 16 hrs. at 7°	.....	7	40
	0.2 g. rat liver	58	65
Dialyzed 16 hrs. at 7°	.....	0	65
	0.2 g. rat liver	42	65

\* 1 ml. of extract was incubated at 37°. The experiment was started through addition of a mixture of 0.32 mg. of adenylyl polyphosphate phosphorus, 88  $\gamma$  of sulfanilamide, and fluoride to 0.05 *M* final concentration.

metal salts, the activator shows the general characteristics of a nucleotide. The presence of phosphate is furthermore indicated by inactivation of coenzyme fractions through incubation with highly purified (66) intestinal phosphatase. The compound is quite sensitive to strong acid and alkali, even at room temperature.

A number of common characteristics link the sulfanilamide acetylation system and the enzyme responsible for reaction between adenylyl pyrophosphate and acetate. Both enzyme systems were found in pigeon liver extracts, possibly because the conditions for a survival of such a system are most favorable there. Both are inactivated by autolysis and dialysis and may be reactivated by heat-stable liver fractions (Tables X and XIII). The acetylation system is, however, more easily reactivated, for instance with boiled liver juice, which only partially reactivates the phosphorylation. It still remains to be decided whether or not the cofactors are identical in both cases.

**Acetylation of Choline.**—During the analysis of acetylation of sulfanilamide, the similarity between it and the acetylation of choline became increasingly evident. The enzyme system responsible for this acetylation has been studied thoroughly by Nachmansohn and his collaborators (53, 55) and more recently by Feldberg and Mann (17). Mann, Tennenbaum, and Quastel (50) had earlier found that, in brain slices, choline acetylation is dependent on the supply of respiratory energy. Nachmansohn and Machado (55) showed that respiratory energy may be replaced by phosphate bond energy (see 37). Addition of adenylyl pyrophosphate promoted anaerobically acetylation of choline in brain homogenate. Here again, however, acetyl phosphate showed no definite activity, although a partial anaerobic reactivation should have been expected in spite of the

rather active acetyl phosphatase present in these homogenates (54). It is noteworthy that, in this case, the phosphorylated product of the acetyl acceptor, choline, namely phosphorylcholine, was likewise inactive when tested as a possible precursor of acetylcholine.

After short dialysis, reactivation had been found with cysteine (55) and, somewhat less effectively, with citrate or, in some cases, glutamate (53). These effects have now been duplicated with the sulfanilamide system with the exception of that of glutamate (unpublished). A very curious inhibitory action of pyruvate and particularly ketoglutarate

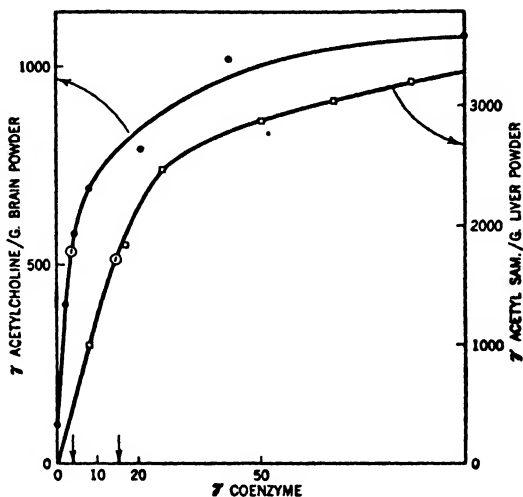


Fig. 10.—Reactivation of acetylation in dialyzed brain and liver extracts by the same coenzyme preparation (41a).

was first described with the choline system. This could not be repeated at first with the sulfanilamide enzyme, but recently it was found that, after prolonged dialysis, ketoglutarate became inhibitory also in the latter system. In general, the liver extract seems to be a better balanced system than the brain extract and must be more severely treated to make the effects of added compounds appear. Likewise, a parallel reaction is the blocking of acetylation by hydroxylamine. Choline acetylation is inhibited by 0.0025, 0.025, and 0.1 *M* hydroxylamine to, respectively, 57, 78, and 93%. As mentioned previously, this inhibition may be interpreted as a trapping of activated carboxyl groups through hydroxylamine, which was shown to react avidly with acetyl phosphate (45).

*Identity of the Coenzyme for Acetylation (41a).*—The apparent analogy of the liver and brain systems made us suspect that the coenzyme, isolated with sulfanilamide acetylation as the test system, may likewise be a component of the brain enzyme. Indeed, experiments with dialyzed pigeon brain extracts have shown that the same coenzyme is active in choline and in sulfanilamide acetylation in the respective enzyme systems of brain and liver. In Figure 10 the parallel reactivation of both reactions with increasing



amounts of the same coenzyme preparation is illustrated. The two curves show that the affinity of the coenzyme apparently is greater in the brain than in the liver enzyme. The relative activities remained constant with various steps of purification.

We have so far not extended our studies to the enzymic mechanism of amino acid acetylation. The recent work with isotopic acetate and acetyl derivatives by Bloch and Rittenberg (7a) suggests some difference between the mechanisms of acetylation of amino acid and of sulfanilamide and choline. Of particular interest is their observation of a transacetylation between various amino acids, as contrasted with exclusive use of free acetate as a source for acetyl sulfanilamide (see also 41). This transacetylation scheme is likely to revive vigorously a discussion of the role of acetylation in peptide formation as proposed by du Vigneaud and Irish (74a).

#### IV. Some General Aspects of the Acetyl Problem

In the theory of the mechanism of acetylation, apparently contradictory facts are harmonized by partial renunciation of the classical concept of a rigorous subdivision of enzymic chains into isolatable intermediaries. The function of adenyl~ph in acetylation thus becomes similar to that of a condensing agent in many organic reactions, and the present problem is reminiscent of the old controversy about the true intermediary in Claisen condensations, *e. g.*, two ethyl acetate molecules to ethyl acetoacetate with sodium ethylate, or the controversy about the role of acetic anhydride in Perkin's reaction of benzaldehyde with acetate to form cinnamic acid. The organic chemical examples mentioned lead into the domain of carbon-to-carbon linkage. The value of acetyl phosphate as a biological reagent in such reactions is evidenced by the enzymic linking, in *Escherichia coli*, of acetyl phosphate and formate to pyruvate, with the elimination of inorganic phosphate. The recognition of acetyl groups as building blocks for straight carbon chains (63) as well as ring structures (5) makes the enzymic mechanism for the lining up of acetic acids into carbon rows a very real problem. Tests with added acetyl phosphate have not been encouraging (32, 60), but for the reasons discussed above such tests do not eliminate it as a possible intermediary, all the more since phosphate (52) and adenyl pyrophosphate (31) have been found necessary for fatty acid oxidation. In order to obtain a more solid theoretical background we shall attempt, in the following section, to calculate the change in free energy in one of the key reactions (77)—the synthesis of acetoacetate from two molecules of acetate.

**Calculation of Change in Free Energy with Acetoacetic Acid Synthesis by Condensation of Two Acetic Acid Molecules.**—Reliable data on the free energy of formation are available for acetic acid and, of course, for water. The  $\Delta F_0$  for acetoacetic acid was computed in two ways: (1) by

using the known value for acetone and introducing the calculated modification for replacing H by COOH as given by Parks and Huffman (62); and (2) by using the known value for butyric acid and introducing the calculated modification for replacing two hydrogen atoms by ketonic oxygen.

(1)	$\Delta F_0$ , kg.-cal.	(2)	$\Delta F_0$ , kg.-cal.
Acetone	- 37.2	Butyric acid	- 91.5
H replaced by COOH	- 83.2	C=O for CH <sub>2</sub>	- 30.0
Acetoacetic acid	-120.4	Acetoacetic acid	-121.5

The close agreement between the two independently computed values is reassuring. Since the modification value for introducing the ketonic group in (2) is considered less reliable (Parks and Huffman, page 211), (1) was used for the following calculations. The free energies of formation for the reactants are assembled in Table XIV.

TABLE XIV  
FREE ENERGIES OF FORMATION

Compound	$\Delta F_0$ , kg.-cal. per mole	Compound	$\Delta F_0$ , kg.-cal. per mole
CH <sub>3</sub> COOH, fluid.....	- 94.5	CH <sub>3</sub> COCH <sub>2</sub> COO <sup>-</sup> , aq.....	-115.6
CH <sub>3</sub> COOH, aq.....	- 96.2	H <sub>2</sub> CO <sub>3</sub> , aq.....	-140.0
CH <sub>3</sub> COO <sup>-</sup> , aq.....	- 89.7	HCO <sub>3</sub> <sup>-</sup> , aq.....	-148.8
CH <sub>3</sub> COCH <sub>2</sub> COOH.....	-120.4	H <sub>2</sub> O, fluid.....	- 56.6

A preliminary evaluation can be obtained by using values for the reactants in fluid state:

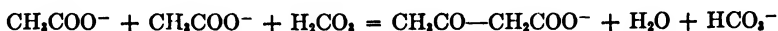


$$\Delta F_0 = -120.4 + (-56.6) - 2(-94.5) = +12 \text{ kg.-cal.}$$

This calculation indicates that condensation of two molecules of acetic acid to acetoacetic acid requires a considerable amount of energy. To approximate more closely the conditions in a living organism, the calculation must be carried out for aqueous solution at neutral reaction. Then the acids are present as carboxylate ions, and, in the condensation, one acetate ion delivers its charge into the buffer reservoir of the medium.

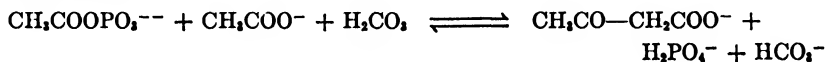
Fortunately, the free energy of formation of acetate is known and that of the acetoacetate ion may be computed fairly reliably by assuming that the difference between free acid (fluid) and carboxylate ion (aq.) will be nearly the same as that between acetic acid (fluid) and acetate ion (aq.), or approximately +4.8 kg.-cal. (see Table XIV). To account for the change

in free energy with the accompanying neutralization, it is most convenient to have the reaction occur in a bicarbonate buffer. The values for the energy change with formation of carbonic acid and bicarbonate ion were taken from Lewis and Randall (33):



$$\Delta F_s = -115.6 + (-56.6) + (-140.0) - (-2 \times 89.7 - 148.8) = +16 \text{ kg.-cal.}$$

Without making claim to high accuracy, this calculation indicates roughly the energy changes involved in the condensation. It is noteworthy that the 16 kg.-cal. required for condensation of two acetates could be supplied, provided that, instead of free acetate, acetyl phosphate reacts with a second mole of acetate. With about -15 kg.-cal. available from the acetyl~ph bond, the reaction:



$$\Delta F_s = +16 - 15 = +1 \text{ kg.-cal.}$$

becomes a reversible reaction, analogous to the reversible condensation of acetyl phosphate and formate to pyruvate.

This equation is written for a pH of 7, where the increase in the second dissociation constant, from inorganic  $pK'_2$  6.9, to acetyl phosphate,  $pK'_2$  4.7, compensates approximately for the deionization of the acetate ion. The  $pK'_2$  of acetyl phosphoric acid was obtained by potentiometric titration (unpublished work).

The calculation shows rather unambiguously that considerable energy is required to link two acetate molecules by elimination of water. This result is supported experimentally through the finding, in liver slices, of a dependence of such a condensation on the supply of respiratory energy (77). A further clue to the mechanism of this activation is furnished through observation (77) of an unequal distribution of  $\text{C}^{13}$  in acetoacetate, synthesized in the presence of acetate with  $\text{C}^{13}$  in the carboxyl group. Less  $\text{C}^{13}$  appeared in the carbonyl, the point of junction between active acetate carboxyl and the methyl group of another acetate, than in the carboxyl group of acetoacetate, which originally had belonged to the "other" acetate (see equations on this page). This is interpreted to show that metabolism generates active acetate more easily through its own breakdown channels than through an activation of added acetate.

At present it appears unwise to attempt a more detailed and necessarily speculative interpretation of the situation. Rapid progress in this field promises an early experimental elucidation of the acetyl question.

## Addendum

*Recalculation of the Change of Free Energy with Hydrolysis of Phosphopyruvate*

Dr. Crawford F. Failey had called my attention to an error in the calculation which appeared in Volume I of this series (37). The energy equation for the split of phosphopyruvate, as energy equivalent with phosphoglycerate, was solved by breaking it into two partial equations: (1) hydrolysis of phosphoglycerate, and (2) dehydration of glycerate to pyruvate. In the calculation of the entropy change in reaction (2),  $S(\text{H}_2\text{O}) = 15.9$  (21) was omitted. By adding  $S(\text{H}_2\text{O})$ ,  $\Delta S_2$  becomes 21.7 instead of 5.8 and:

$$\Delta F_2 = -\Delta H_2 - T\Delta S_2 = -6500 - 298 \times 21.7 = -12,950 \text{ cal.}$$

With a  $\Delta F_1$  for splitting of an "energy-poor" phosphate ester, of about 3000 cal., the sum  $\Delta F_1 + \Delta F_2$  now approximates  $-15,950$  cal.

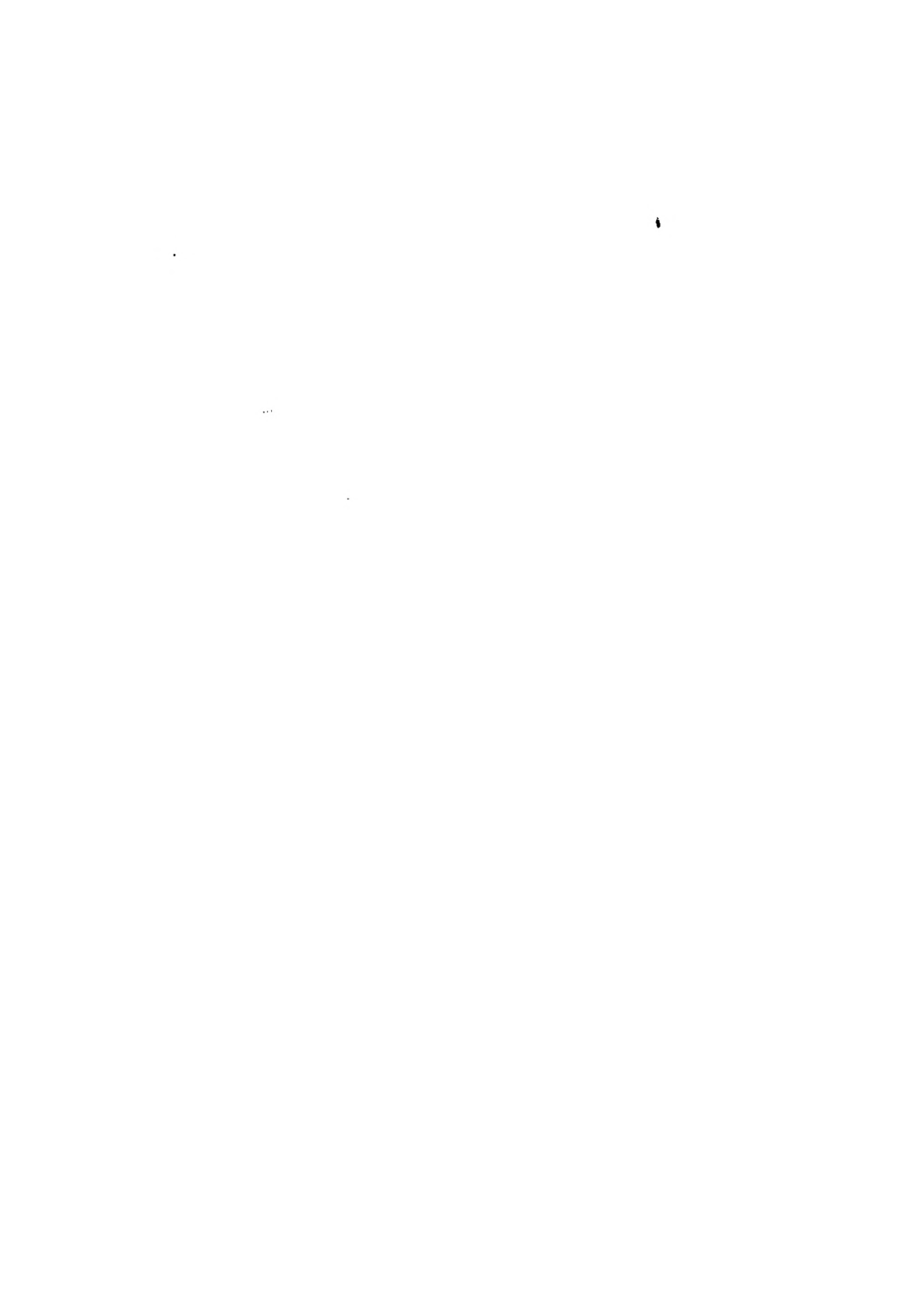
The amended value of nearly 16 kg.-cal. for the split of phosphopyruvate conforms fairly well with Lardy's data (29) on the transfer of adenyl~ph to pyruvate which indicate a bond energy in ph~pyruvate of about 3 kg.-cal. above that in adenyl~ph.

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# MICROBIAL ASSIMILATIONS

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## I. Introduction

The complexity of the biochemistry of living matter manifests itself to the greatest extent in the synthetic processes, which must be provided for as growth and multiplication occur. A so-called "simple" microorganism, such as an autotrophic bacterium, may increase in numbers a millionfold during a short period of time in a solution of inorganic salts, with carbon dioxide serving as the carbon source. Other microorganisms may require the addition of one or more organic compounds to the medium before multiplication becomes evident; yet in either case, cells are formed with an array of structural matter, enzymes, vitamins, and other regulatory substances of a complexity as striking as that observed in higher forms of life.

It has been generally assumed that oxidation of the foodstuff made energy available to the cell for synthetic processes, yet the energy coupling between the reactions involved in catabolism and anabolism was difficult to picture in either physical or chemical terms. More recently the interpretation of the mechanism of many catabolic processes has led to a desire



to interpret the anabolic processes in a similar manner. The conversion of lactate, water, and ammonia into proteins, fats, carbohydrates, and other cellular constituents must obviously be a complex series of chemical reactions in the same sense as is the catabolic breakdown of glucose to lactic acid, to carbon dioxide and alcohol, or to carbon dioxide and water. The essential differences are that the former reactions are far more difficult to study and, in addition, do not proceed spontaneously, *i. e.*, free energy must be available.

Studies with microorganisms have suggested that there is not only an intimate, but also a quantitative relationship between the main phases of the catabolic and anabolic processes. Studies on the mechanism of the catabolic reactions have furnished information concerning the intermediate degradation products and do suggest that biological syntheses may ultimately be expressed as series of consecutive step reactions involving, at least in part, intermediates formed during the stepwise degradation of the foodstuff molecules. It is the purpose of this review to consider the studies dealing with the assimilatory processes of nonphotosynthetic microorganisms, and to attempt as far as possible to correlate observations on the relation between the anabolic and catabolic activities of microbial life.

## II. A Concept of Assimilation

In the synthetic processes we are dealing with reactions in which one or more products of the reaction gain in free energy. This is made possible by simultaneously occurring reactions in which the free energy decreases, the free-energy increase of the products of synthesis being less than the free-energy decrease of the system as a whole. An example is the resynthesis of glycogen (65) during the aerobic recovery phase after muscle contraction, the resynthesis of glycogen from lactic acid taking place at the expense of the simultaneous combustion of another portion of the lactic acid. From the viewpoint of free energy change of the system as a whole, the resynthesis of glycogen is entirely feasible. However, no satisfactory explanation was advanced as to the mechanisms involved in the transfer of the energy liberated during the combustion of lactic acid to the energy-requiring synthetic processes leading to the resynthesis of glycogen. Moreover, lactic acid is markedly different in structure from glycogen; and lactic acid, or products obtained therefrom, must undergo a number of rearrangements and condensations before the resynthesis of glycogen is completed.

Kluyver (47) pointed out that the possibility of synthesis is inherent in the nature of oxidation-reduction reactions of the type  $AH + B \rightarrow A + BH$ , in which, although there

is a decrease in total free energy, the energy content of BH is greater than that of B, while at the same time the energy content of AH is degraded to that of A. In other words, BH can be regarded in the broadest sense as a product of a synthesis occurring during the spontaneously proceeding reaction  $AH + B \rightarrow A + BH$ , since the free energy of BH is greater than that of the substance from which it was derived. In a synthesis of this sort it is unnecessary to postulate a transfer of energy between two materially independent reactions.

Likewise, Kluver pictured the synthesis of an amino acid from a keto acid and ammonia as a series of reactions in which ammonia is taken up in a reversible spontaneous reaction by the keto acid. The intermediate compound thus formed acts as a hydrogen acceptor in a coupled oxidation-reduction of the type  $AH + B \rightarrow A + BH$ , and is thereby reduced to the product of synthesis, an amino acid.

The resynthesis of glycogen could be explained on a similar basis as involving a partial oxidation of the lactic acid to pyruvic acid, the latter then undergoing an intramolecular oxidoreduction in which the keto group gains in energy, *i. e.*, is reduced. At the same time the carboxyl carbon is oxidized and split off as carbon dioxide. The acetaldehyde formed by the decarboxylation of pyruvate could be further oxidized to an intermediate such as glycol aldehyde, which in turn could be converted into carbohydrate by a chain of spontaneous reactions. The value of this concept was that it illustrated a possible mode of synthesis of a product of higher energy and carbon content from a simpler compound, without energy transfer from a materially independent reaction.

Kluver also slightly modified the hypothetical scheme for the synthesis of fat by *Endomyces vernalis* as advanced by Haehn and Kintof (41) to illustrate that the synthesis of fat could be explained on a basis similar to that of carbohydrate synthesis. Acetaldehyde formed during the oxidative utilization of carbohydrate could undergo an aldol condensation with the formation of crotonic aldehyde. Crotonic aldehyde after reduction to butyraldehyde could undergo an aldol condensation with acetaldehyde or butyraldehyde. Thus a series of aldol condensations and reductions at the double bond could lead to the formation of the higher aldehydes which in turn could be oxidized to the corresponding fatty acids. Again, synthesis was regarded as a series of spontaneous reactions leading to the formation of a compound or compounds with higher energy content and was accompanied by a concomitant decrease in the free energy of the system as a whole.

Furthermore, compounds such as butyric acid are frequently formed during the course of bacterial decompositions of carbohydrates. Butyric acid may also be formed from acetaldehyde added to the fermentation mixture. In the first case we might postulate butyric acid to be a product split from the carbohydrate molecule during the course of its degradation. In the latter case it must be a product of synthesis although it is excreted by the cell and cannot strictly be regarded as a product of an assimilatory reaction. Similar reactions could conceivably lead to the formation of true assimilatory products. In either case acetaldehyde, or some compound derivable therefrom, could be regarded as the precursor of butyric acid. Therefore, the latter must be regarded

as a product of synthesis, even though in one case it is ultimately formed from a substrate of higher energy content; in the other from a substrate with lower free energy.

Although certain of the intermediate compounds and reactions proposed by Kluyver may not be tenable on the basis of our present knowledge, this basic concept of synthesis and assimilation may well merit further consideration. Such a concept will serve as a guide in this discussion, and with it as a foundation we may define synthesis in the broadest sense as the formation of a compound with a higher free energy content than the immediate precursor molecule from which the product of synthesis was derived. Assimilation can then be regarded as either partial or total utilization by the cell of a product of synthesis which is of value in the economy of the cell. Thus we see that synthesis may occur with or without partial or complete assimilation of the synthesized material by the cell. In fact, on the basis of our present knowledge of assimilatory processes, it may be impossible to differentiate at times between synthesis and assimilation. Likewise, it may become evident that there is no sharp line of demarcation between catabolic and anabolic processes, the chains of reactions leading to the synthesis of cellular material having their origins in one or more stages of the dissimilatory process and being intimately and energetically coupled therewith.

### III. Assimilation of Carbon

Wilson and Peterson (90) concluded that the amounts of energy liberated during the growth of bacteria may range from two to one hundred times the values reported for the energy content of the cells. The early studies on the energetics of bacterial growth are complicated by the fact that the studies were of relatively long duration and, therefore, much of the expended energy might be considered as "energy of maintenance" derived either from the substrate or from reserve food materials synthesized by and stored in the cells. Free energy efficiencies calculated from the results of such studies do not necessarily give a true index of the efficiency of the basic assimilatory process. A clearer insight into the fundamental assimilatory process could be obtained from studies of short duration and under carefully controlled conditions.

With nonproliferating cells, it is not generally appreciated to what an extent assimilation may occur. Fürth and Lieben (33) found that about one-half of the lactic acid utilized in an aerated yeast suspension was converted to a "hydrolysis-resistant carbohydrate." The following year Lieben (57) reported that pyruvate is in part decomposed with carbon dioxide production, in part assimilated with the formation of "body substance." This observation was confirmed and extended by Smedley-Maclean and

Hoffert (73) who reported that both the fat and carbohydrate content of yeast cells increased during the course of oxidation of pyruvate, lactate, acetate, or ethanol. In nearly all these studies, however, it should be noted that the experiments ran for a considerable number of hours. Carbohydrate synthesis by animal tissues was also observed by a number of workers. Takane (80) showed that liver slices synthesized carbohydrate from lactate, while Bach and Holmes (4) demonstrated synthesis from lactate and pyruvate. Benoy and Elliott (8) observed carbohydrate synthesis from lactate and pyruvate in rat liver slices; and synthesis from lactate, pyruvate, succinate, fumarate, malate, and alanine by kidney cortex.

Cook and Stephenson (20), in experiments of relatively short duration on the oxygen uptake by washed suspensions of *Escherichia coli*, found that the oxidation of formate is carried to completion, while the oxygen uptake with glucose or lactate amounted to two-thirds, and with acetate to three-fourths of the amounts required for complete oxidation. A similar behavior was noted with suspensions of *Alcaligenes faecalis* with the exception that glucose was not oxidized by this organism. Cook and Stephenson were unable to demonstrate that the oxidations ceased because of (a) enzyme destruction, (b) threshold concentrations being reached below which oxidations could not occur, or because of (c) the accumulation of nonutilizable end products of oxidation. The rate and extent of these oxidations did not appear to be dependent on the proportion of living cells, *i. e.*, those capable of multiplication on transfer to nutrient media. Similar manometric experiments by Lineweaver (59) appeared to indicate that *Azotobacter vinelandii* oxidized various substrates to completion. Lineweaver's experiments were of twelve to thirty hours' duration, and an examination of his results indicates that marked reductions in rates of oxygen consumption occurred within the first few hours. The slower rates of oxygen uptake observed on longer standing may well have been due to an endogenous respiration.

The studies of Barker (5) on the oxidation of a number of simple organic compounds by *Prototheca zopfii* (a colorless alga) constitute a marked advance in the study of assimilation. Barker observed that the amounts of oxygen consumed in manometric experiments of one to four hours' duration varied with the nature of the compound, but that no proportionality existed between the amounts required for complete combustion and the experimentally observed oxygen consumptions. With glycerol as a substrate the oxygen consumption was 29% of the theoretical value for complete combustion; corresponding percentages for other substrates being 46 for ethyl alcohol, 30 for glucose, 50 for acetic acid, 58 for propionic, 48 for *n*-butyric, 58 for isobutyric, and 33 for *n*-valeric acid. Barker's results on oxygen consumption and carbon dioxide production are summarized in Table I where the oxygen consumed and carbon dioxide produced are expressed as moles per mole of substrate utilized. The observed values are compared with the theoretical values as calculated for complete oxidation to carbon dioxide and water. No correction was made for the respiratory exchange of the cell suspensions without added substrate. He presented some evidence that the endogenous respiration might be reduced in

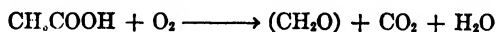
the presence of a readily utilizable substrate, but the application of an accurate correction for endogenous respiration remains as an unsolved problem (see Winzler, 91). However, if a correction for endogenous respiration were employed, it would mainly tend to reduce the values for oxygen consumption and carbon dioxide production during the utilization of the substrate, and so increase the percentage of substrate not oxidized to carbon dioxide and water.

TABLE I  
OXIDATION OF ORGANIC COMPOUNDS BY *Prototheca zopfii* (5)

Substance	Oxygen consumed*		Carbon dioxide produced*		Respiratory quotient	
	Theor.	Obs.	Theor.	Obs.	Theor.	Obs.
Glycerin	3.50	1.01	3.00	0.62	-0.86	-0.61
Ethyl alcohol	3.00	1.37	2.00	0.49	-0.67	-0.36
Glucose	6.00	ca. 1.8	6.00	1.98	-1.00	ca. -1.1
Acetic acid	2.00	1.00	2.00	1.03	-1.00	-1.03
Propionic acid	3.50	2.03	3.00	1.56	-0.86	-0.77
n-Butyric acid	5.00	2.40	4.00	1.70	-0.80	-0.71
Isobutyric acid	5.00	2.90	4.00	1.94	-0.80	-0.67
n-Valeric acid	6.50	2.14	5.00	1.63	-0.77	-0.76

\* Oxygen and carbon dioxide are expressed as moles per mole of substrate decomposed. The theoretical values are calculated for complete oxidation to carbon dioxide and water.

Qualitative tests for the presence of various oxidation products, other than carbon dioxide and water, in experiments on a larger scale were negative, except for small quantities of lactic acid. In one experiment 89% of the substrate, acetic acid, was accounted for as carbon dioxide and cellular material. Manometric experiments on the decomposition of acetate indicated that only one molecule of oxygen was taken up per mole of acetic acid utilized, while two moles are required for complete oxidation, and also one instead of two molecules of carbon dioxide was produced. Hence, it is possible to deduce the empirical composition of the product (s) from the experimental data, just as in the process of photosynthesis the formation of a carbohydrate is postulated from the value of the photosynthetic quotient. Applying this mode of reasoning to the utilization of acetic acid gives:



as a balanced equation for the reaction. The theoretical values for oxygen consumption or carbon dioxide production for this postulated reaction is one mole per mole of acetic acid decomposed, while the experimentally determined values were 1.00 for oxygen consumed and 1.03 for carbon dioxide produced.

In the same manner, equations based on the data presented in Table I were derived to represent the oxidation of the other substrates. These equations are presented in Table II, together with the theoretical gas exchange based on these equations and the gas exchange actually observed. It is apparent that the agreements between the experimental results and those based on the theoretical considerations are excellent with acetic, propionic and isobutyric acids, and with ethyl alcohol as substrates; fair with butyric acid and glycerol; and poor with valeric acid.

TABLE II  
OXIDATIVE SYNTHESIS OF CARBOHYDRATES BY *Prototheca zopfii* (5)

Substance	Conversion	Oxygen consumed*		Carbon dioxide produced*	
		Theor.	Obs.	Theor.	Obs.
Glycerin	$2 (C_3H_8O_3) \rightarrow (CH_2O)_5$	1.00	1.01	0.50	0.61
Ethyl alcohol	$2 (C_2H_6O) \rightarrow (CH_2O)_2$	1.50	1.37	0.50	0.49
Acetic acid	$(C_2H_4O_2) \rightarrow (CH_2O)$	1.00	1.00	1.00	1.03
Propionic acid	$2 (C_3H_7O_2) \rightarrow (CH_2O)_3$	2.00	2.03	1.50	1.56
n-Butyric acid	$2 (C_4H_8O_2) \rightarrow (CH_2O)_5$	2.50	2.40	1.50	1.70
Isobutyric acid	$(C_4H_8O_2) \rightarrow (CH_2O)_2$	3.00	2.90	2.00	1.94
n-Valeric acid	$2 (C_5H_{10}O_2) \rightarrow (CH_2O)_7$	3.00	2.14	1.50	1.63

\* Oxygen and carbon dioxide are expressed in moles per mole of substrate decomposed.

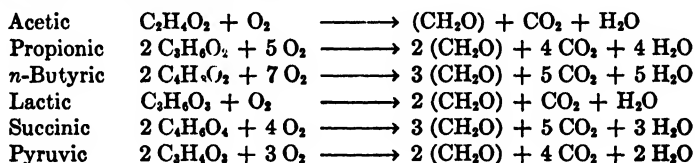
The data presented by Barker suggest that a carbohydrate (probably glycogen since glycogen is stored by *P. zopfii*) is the primary product of oxidative assimilation, but do not offer an explanation of the mechanism by which the various substrates are converted. Acetic acid could be oxidized via glycolic and glyoxylic acids or via succinic and fumaric acids, but none of these compounds are attacked by *P. zopfii*.

Anderson (3a) observed that acetic acid does not appear to be oxidized via glycolic acid by *P. zopfii* since the oxygen consumption observed in the presence of glycolic acid was generally two to three times that required for complete combustion. The addition of glycolic acid to a suspension of *P. zopfii* appeared to stimulate the endogenous respiration of the cells and Anderson's observations suggested that glycolic acid might function as a respiratory catalyst. Glyoxylic acid, on the other hand, was respired in the normal manner. Thus it appears that acetic acid is not oxidized by way of glycolic acid.

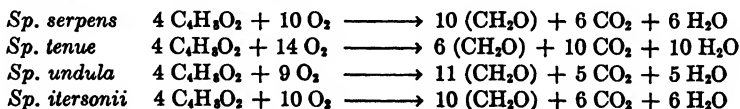
Anderson also observed that pyruvic and lactic acids could be oxidized by *P. zopfii* in an acid medium. The addition of thiamin to thiamin-deficient cells stimulated the oxidation of lactic and pyruvic acids, glycerol, and glucose, but not that of acetate. The discovery of the ability of *P.*

*zopfii* to oxidize lactic and pyruvic acids in an acid medium led to further studies on oxidative assimilation by this organism. Anderson found that assimilation occurs to the same extent with pyruvic acid as with lactic acid and concluded, in agreement with the observations of Clifton and Logan (16) and of Doudoroff (25) to be discussed later, that there is a relatively simple chemical mechanism of assimilation rather than a strictly energetic coupling between anabolic and catabolic reactions.

The studies of Giesberger (38) on the oxidative utilization of various organic compounds by different species of *Spirillum* lend further support to the theory of oxidative assimilation as advanced by Barker. Giesberger observed that hydroxy, keto, and dibasic acids as well as the simpler acids and alcohols were not oxidized to completion by the different species studied. From the experimentally determined values for oxygen consumption and for carbon dioxide production during the utilization of a known amount of substrate, Giesberger established carbon and oxygen balances which led to the same type of equations as postulated by Barker (5) for oxidative assimilation by *P. zopfii*. With *Sp. tenue*, for example, the experimental results with various acids suggested that the oxidative assimilation might be expressed as follows:



In addition, he pointed out that assimilation may occur to different extents from the same substrate but with different species of the genus *Spirillum*. The experimental data on the oxidation of butyric acid by different species suggested the following equations:

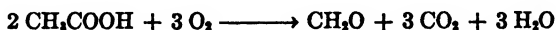


These differences may be more apparent than real since these equations are only approximations; the endogenous respiration of one species may be reduced to a greater extent than that of a second in the presence of added foodstuff, or more by-products may accumulate in the medium.

More direct evidence that assimilation did occur during respiration was obtained on comparison of photomicrographs of stained cells before and after the utilization of a known amount of substrate. The actively re-

spiraling cells showed an increased volutin content as judged by marked increase in numbers of intracellular volutin granules. No marked increase in volutin content was observed when the suspension medium was water rather than phosphate buffer, thus suggesting that phosphorus was essential for volutin formation. That volutin formation occurs to a considerable extent in the absence of an extraneous nitrogen source is somewhat surprising, since volutin consists in part of nucleoprotein. Giesberger did note, however, that assimilation may occur without an observable increase in volutin granules, and that volutin represents but one of the assimilation products. Certain of his observations also suggested that an assimilation product somewhat more reduced than carbohydrate may be formed. Thus, it is apparent that the postulated equations for oxidative assimilation represent a resultant of the numerous reactions occurring during the primary assimilatory process.

Clifton (unpublished results) confirmed the studies of Giesberger (38) with *Spirillum serpens* and carried out similar studies with *Pseudomonas calco-acetica* and *Escherichia coli*. He found (15) that the oxidation of acetate by *Ps. calco-acetica* was characterized by a slight lag period, the rate of oxygen consumption rapidly increasing to a relatively constant value which was maintained until approximately two-thirds of the oxygen required for the complete combustion of the acetate had been consumed. Then the rate of oxygen consumption rapidly decreased and by the time three-fourths of the oxygen required for complete combustion had been consumed, the rate approached a level near that of the substrate-free control. Typical results graphically illustrating the rate and extent of oxygen consumption by *Ps. calco-acetica* in the presence of different concentrations of acetate are presented in Figure 1. During the period of rapid oxygen consumption the R.Q. was 1.05 and fell to a level near 0.9 after the rate of oxygen consumption decreased to the level of the control suspension (R.Q. 0.93). Following the reasoning of Barker and of Giesberger, the experimental results suggested that the oxidative assimilation of acetate may be expressed as:



According to this equation three-fourths of the theoretical amount of oxygen required for the complete combustion of acetate is consumed with the production of an equivalent amount of carbon dioxide. Concurrently one mole of a substance with the empirical composition of a carbohydrate is assimilated per two moles of acetate disappearing from the suspension medium. It was assumed that the respiration of the cells as observed in



control suspensions was largely suppressed in the presence of acetate. If, on the other hand, the endogenous respiration proceeds in the presence of an added foodstuff, the R.Q. would not be appreciably altered but the amount of synthesis would be increased by approximately 10% over that postulated by the above equation.

A number of possible explanations for the oxidation falling short of completion were considered. One possibility for the rate of oxygen consump-

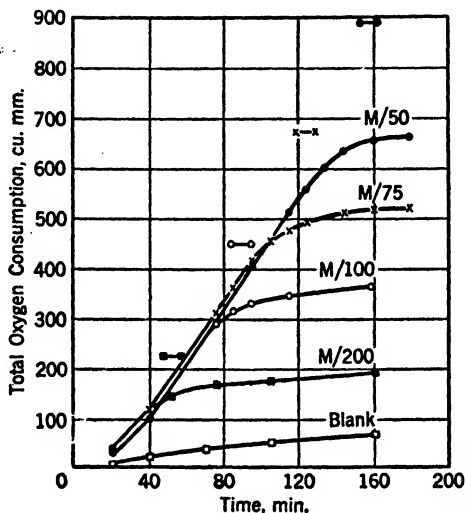


Fig. 1.—Influence of different concentrations of acetate on total oxygen consumption per cubic centimeter of a suspension of *Pseudomonas calco-acetica* (15).

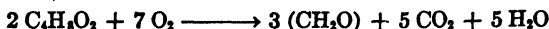
tion decreasing abruptly at three-fourths completion is that the concentration of substrate had decreased to a level below which oxygen consumption does not occur at a measurable rate. This explanation was rendered untenable by results, similar to those presented in Figure 1, observed over a wide range of acetate concentrations. The rates of oxygen consumption decreased to a level near that of the control suspension when 70 to 75% of the theoretical amounts of oxygen had been consumed, irrespective of the initial concentration of acetate. A similar extent of oxygen consumption was observed when the initial concentration of acetate was maintained constant, while the numbers of bacteria in the Warburg flasks were varied over a wide range.

The possibility exists that decomposition products inhibitory to respiration may accumulate in the medium. This was excluded by the addition of more acetate after the break in rate of oxygen consumption was observed. The addition of more acetate resulted in an immediate renewed oxygen uptake at approximately the original rate and to the same extent. The further addition of acetate led to similar results. The addition of a fresh bacterial suspension to the contents of a vessel after the break in rate of oxygen consumption brought about no increase in the rate above that observed in a similar control suspension. These observations exclude the possibility of decomposition products inhibiting respiration, and furthermore exclude any appreciable destruction of enzymic activity of the cells. Since carbon dioxide was removed by absorption with alkali, it could not interfere with oxidation by a mass action effect. Also, the extent of oxygen consumption appeared to be the same in the presence or absence of a carbon dioxide absorbent. Since the change in pH of the suspension during the course of an experiment was negligible, and since the rate and extent of oxygen consumption was approximately constant over a fairly wide range of pH, any inhibitory effect of hydrogen ions was also excluded.

The influence of the concentration of substrate or of bacteria, of the addition of substrate or of organisms during the course of the experiment, and of pH on the rate and extent of oxygen consumption by *Ps. calco-acetica*, *Sp. serpens*, and *E. coli* in the presence of acetate, butyrate, or glycerol was also studied. Essentially the same type of behavior as discussed above was observed. The manometric results therefore suggest that the phenomenon of incomplete combustion does indeed represent a quantitative relationship between assimilation and oxygen consumption, since qualitative tests indicated the complete disappearance of the substrate and no accumulation of typical end products of fermentation or possible intermediate products of oxidative dissimilation. These observations are in complete agreement with those reported by Cook and Stephenson (20).

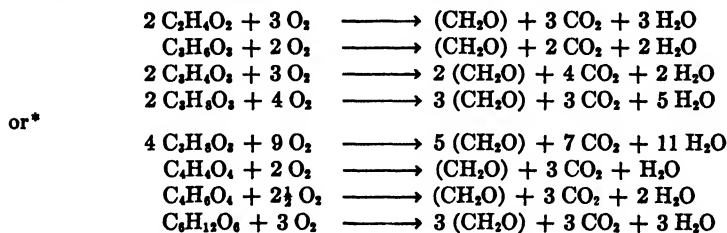
The results obtained with butyrate as a substrate for *Ps. calco-acetica* were of particular interest in that two phases of oxidation, characterized by different R.Q. values, could be observed. When acetate or glucose served as the substrate, the oxygen consumption and carbon dioxide production were approximately equivalent, thus giving rise to an R.Q. of unity. During the phase of oxidative assimilation of butyrate, oxygen was consumed at a rapid and quite constant rate until the oxygen consumption had proceeded approximately 75% to completion. The R.Q. observed during this phase of activity was in the neighborhood of 0.68 (while the R.Q. for complete oxidation of butyrate is 0.80). During an equivalent

period of time after the pronounced break in rate of oxygen uptake was observed, the R.Q. was 0.94, a value characteristic of the respiration of the control suspension. In the presence of appropriate cellular poisons, which will be discussed later, the R.Q. during the phase of rapid oxygen uptake was approximately 0.80. The low R.Q. observed with butyrate lends further support to the concept that assimilation does occur in washed suspensions of bacteria, and in conjunction with the consumption of three-fourths of the oxygen required for complete combustion, suggests that the oxidative assimilation of butyrate by *Ps. calco-acetica* may be represented as:



This equation indicates an oxygen uptake of 70% of the theoretical and an R.Q. of 0.7 (obs., 75% and 0.68).

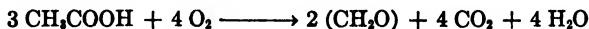
Clifton and Logan (17), from a study of the oxidation in phosphate or bicarbonate buffers of a number of organic compounds by washed suspensions of *E. coli*, concluded that a portion of the substrate was always assimilated by the cells. Indirect evidence in support of this concept of oxidative assimilation was obtained in studies on the growth of *E. coli* in the presence of the same substrates, similar relations being observed between the extent of oxygen consumption and assimilation in actively proliferating cultures and in washed suspensions. The experimental results suggested the following equations for oxidative assimilation of acetate, lactate, pyruvate, glycerol, fumarate, and glucose by *E. coli*:



Winzler and Baumberger (93), using an adiabatic calorimeter, measured the rate of heat production during both aerobic and anaerobic utilization of glucose by *Saccharomyces cerevisiae*. From the heats of formation and the heat produced during exogenous respiration, they concluded that 26.5% of the glucose disappearing from the medium was oxidized to carbon dioxide and water and 75.5% assimilated under aerobic conditions; corresponding figures for anaerobic assimilation being 70.5% fermented to ethyl alcohol and carbon dioxide, while 29.5% was stored. With acetate as the substrate, 58.7% was oxidized while 41.3% was utilized in synthesis.

\* See p. 286 for this modification of the equation representing glycerol assimilation.

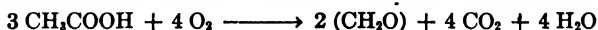
Winzler (91) determined the oxygen uptake, carbon dioxide production, acetic acid utilization, and carbohydrate synthesis by *S. cerevisiae* in the presence of small amounts of acetate. The respiratory exchange indicated the oxidation of two-thirds of the acetic acid to carbon dioxide and water (compared with 58.7% conversion on the basis of heat exchange with a different strain of yeast) and the conversion of one-third of the acetate carbon to cellular material according to the equation:



This relation was independent of the pH between 3.5 and 8, of aeration of the substrate-free yeast for periods as long as 3 days, and of temperature between 20° and 30° C.

A curious behavior was observed on comparison of assimilation by yeast grown on solid and in liquid media. A constant ratio between assimilation and oxygen uptake in the presence of different concentrations of acetate was observed with yeast grown on a solid medium when no correction was applied for endogenous respiration. On the other hand, a constant ratio was observed with yeast from liquid media only when a correction for endogenous respiration was applied to the results of the exogenous respiration experiments. Winzler concluded that yeast cells from the liquid medium maintained their endogenous respiration in spite of the simultaneous oxidation of acetate. When a correction for endogenous respiration was applied to the results obtained with cells from the liquid medium, the same relation between oxygen consumption and assimilation was obtained as indicated in the preceding equation.

Winzler (91) also provided direct evidence that carbohydrate is indeed synthesized by demonstrating an increase in the reducing sugar content, above that observed in the original cells, of yeast after hydrolysis in dilute sulfuric acid for three hours following the phase of rapid assimilation of acetate. In twelve separate experiments the carbohydrate synthesized varied from 56.2 to 117.5% of that postulated by the equation:



the average increase being 80.5% of the theoretical value. The presence of glucose, lactic, succinic, or citric acids, acetaldehyde or ethyl alcohol could not be detected in the supernatant fluids from the Warburg flasks.

Macleod and Smedley-Maolean (64), on the other hand, concluded that acetate is directly condensed to lipoid without first being synthesized to a carbohydrate. Experiments by Sonderhoff and Thomas (75) in which trideuteroacetate was employed as the substrate also tended to support a primary synthesis of fat. After incubation in trideuteroacetate, 14.7% of the total lipid hydrogen was deuterium while only 1.6% was deuterium in the case of carbohydrate. These experiments were generally

of longer duration and carried out in the presence of higher concentration of acetate than employed in Winzler's studies. It may well be that if analysis had been made earlier in the course of incubation, a primary synthesis of carbohydrate would have been observed. Stier and Newton (78), as will be discussed later, observed an initial storage of carbohydrate from glucose followed by a phase of fat (ether-extractable material) storage.

Fink, Krebs, and Lechner (29) observed that yeast oxidizes glucose with the production of only two molecules of carbon dioxide per hexose molecule utilized, fermentation being almost completely suppressed under well-aerated conditions. The other four carbon atoms were accounted for as increased cellular material of the empirical composition  $(CH_2O)_x$ . This oxidative assimilation was expressed as:



The authors considered acetaldehyde to be the turning point between dissimilation and synthesis. In macrogrowth experiments, 90% of carbon, postulated to be assimilated by the above equation, was actually recovered as cellular carbon in cultures of *Torula utilis*.

Van Niel and Anderson (85) reported that equimolar quantities of ethyl alcohol and of carbon dioxide are produced during the fermentation of glucose, levulose, mannose, sucrose, or maltose by *S. cerevisiae*, the two products together accounting for only 70% of the sugar added to the suspensions. This is in very close agreement with the results (70.5%) for heat production observed by Winzler and Baumberger (93) with glucose as the substrate. Van Niel and Anderson also demonstrated an increase in dry weight of both the cells and of the supernatant liquid during the course of the fermentation of glucose, typical results being presented in Table III.

TABLE III

DRY WEIGHT (IN MILLIGRAMS) OF CELL MATERIAL AND OF SUPERNATANT RESIDUE OF *Saccharomyces cerevisiae* SUSPENSIONS AFTER FERMENTATION OF DEXTROSE (85)

Analysis	Expt. I	Expt. II	Expt. III
Dry weight of yeast:			
Experimental	16.907	18.821	31.258
Control (no dextrose)	13.447	17.239	28.508
Difference	3.460	1.582	2.750
Dry weight of supernatant residue:			
Experimental	5.313	7.714	12.708
Control	4.253	6.854	9.818
Difference	1.060	0.860	2.990
Total increase in dry weight:			
Yeast and supernatant	4.520	2.442	5.740
Dextrose used (not accounted for as $CO_2$ and ethanol)	4.32	2.09	5.91
Per cent recovery	104.7	116.0	97.2

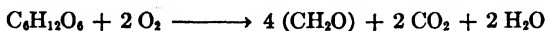
These results definitely indicate an increase in dry weight of the cells during the course of fermentation, and lend further support to the concept that assimilation may occur during the utilization of a single substrate by washed cells under either aerobic or anaerobic conditions. However, it should be noted that no fermentative assimilation could be observed during lactic acid fermentation of glucose or levulose by *Streptococcus faecalis*, as determined by carbon dioxide production in bicarbonate buffer. This behavior should be studied in more detail. Van Niel and Cohen (86) later observed fermentative assimilation by *Candida albicans* (see also (39)).

Stier, Newton, and Sprince (79) observed an increase in opacity, of volume, and of dry weight per unit number of cells during a six-hour period of incubation in 5% glucose. Stier and Newton (78) reported that the rate of oxygen consumption of bakers' yeast was constant for about the first two hours in a 5% dextrose solution and that it then steadily declined during the next hour, finally becoming constant again but at a lower rate. The R.Q. during the first period was 1.3 and after 240 minutes had decreased to 1.0. Another strain of bakers' yeast exhibited a smaller decrease in rate of oxygen consumption at about 200 minutes after the addition of glucose, the R.Q. being 1.44 before the shift and 1.66 during the latter period. They postulated that the shift in respiratory rates was due to a "partial block" of some enzyme surface involved in the respiratory and assimilatory processes, possibly produced by the accumulation of extracellular by-products of glucose metabolism. The observed changes in rate of respiration were also correlated with changes in anabolic activity of the cells. During the first phase of high respiratory activity, a very rapid rate of increase in dry weight of the cells was noted. This increase in dry weight resulted primarily from an increase in carbohydrate content of the cells. During the second constant-rate phase, the dry weight continued to increase but at a much slower rate and with no further demonstrable increase in total carbohydrate. Stier (77) reported that dissimilated yeast (aerated three days in  $M/15$   $\text{KH}_2\text{PO}_4$  at  $25^\circ\text{C}$ .) synthesized considerably more glycogen from glucose under anaerobic than under aerobic conditions. With fresh yeast, glycogen synthesis from glucose was practically the same under either aerobic or anaerobic conditions.

These results further support the theory of oxidative assimilation but are difficult to interpret, in that Stier and Newton (78) reported an R.Q. of 1.3 during the rapid phase of carbohydrate storage. However, inspection of Figure 1 in the paper of Stier and Newton suggests that, in the particular experiment recorded therein, the R.Q. was close to unity at the end of 130 minutes. It may well be that a considerable portion of the

glucose was fermented, thereby markedly increasing carbon dioxide production and the observed R.Q. during the phase of rapid oxygen consumption. Strict aerobic assimilation with the formation of carbohydrate from carbohydrate would be occurring only during respiration proceeding with an R.Q. of unity

Pickett and Clifton (67) studied the oxidative assimilation of glucose by *S. cerevisiae* in considerable detail and found that approximately one-third of the total amount of oxygen required for complete combustion of glucose was consumed during the phase of rapid oxygen consumption. This, together with an observed R.Q. of unity, suggested that the oxidative assimilation of glucose by this particular strain of yeast could be represented as:



Determinations of increase in readily hydrolyzable carbohydrate or in dry weight of the cells accounted for only about one-half of the carbon postulated as assimilated by the above equation. The above equation postulates 66.6% assimilation of glucose carbon, while in a number of experiments the per cent of carbon recovered as reducing carbohydrate varied from 21.5 to 41.7% and as increase in dry weight from 20.8 to 40.3%, the average values being 30.2 and 29.7%, respectively. When the values were corrected for residual glucose in the suspension medium, the average recoveries were 37.6% on dry weight basis and 32.4% on the basis of carbohydrate content of the cells. Since increases in carbohydrate content and dry weight of the cells were roughly equivalent, it does not appear that fat synthesis is proceeding to any marked extent and little or no change in ether-extractable material was observed experimentally.

Attempts were made to determine the nature of the materials derived (or synthesized) from glucose but not assimilated by the cells. Qualitative and quantitative tests were carried out for possible end products of glucose metabolism and, in particular, those substances which could be derived from glucose but are not readily utilized by yeast. In one experiment 31.0% of the glucose carbon was accounted for as carbon dioxide 29.5% as increase in carbon content of the cells, 29.2% as nonvolatile matter and 7.8% as carbon in volatile organic matter, a total recovery of 97.5%. In parallel experiments the suspension medium was found to contain 1.4% of the carbon as reducing sugar, 0.7% as neutral lipide, 2.5% as glycerol, 0.1% as hexose diphosphate, and 2.8% as succinic acid, a total of 7.5% as compared with a total nonvolatile carbon content of 29.2% in a similar experiment. Also 1.5% of the glucose carbon was recovered as ethyl

alcohol and 1.0% as acetic acid, as compared with a total of 7.8% on the basis of microcombustion of the volatile matter. Tests for acetylmethylcarbinol, 2,3-butyleneglycol and pyruvic, lactic, fumaric, gluconic, citric, phosphoglyceric, and glycerophosphoric acids were negative.

Van Niel and Anderson (85) observed (see Table III) both an increase in dry weight of the cells and of the suspension medium after the anaerobic utilization of glucose by yeast. No search was made for products of fermentation other than ethyl alcohol and carbon dioxide, but it is apparent that under both aerobic and anaerobic conditions only a portion of the glucose postulated to have been assimilated on the basis of manometric studies actually is assimilated. It is quite possible that a considerable portion of the nonassimilated carbon has actually been involved in synthetic reactions but has not been assimilated by the cells, *e. g.*, the synthesis of "yeast gum."

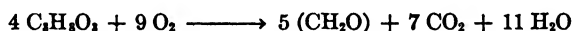
With other organisms the actual extent of assimilation may be closer to that postulated from the results of manometric experiments. Clifton and Logan (16, 17) observed approximately the same relation between the extent of oxidation and assimilation in actively proliferating cultures as in washed suspensions of *Escherichia coli*. Whelton and Doudoroff (89) observed that the average amounts of assimilation in cultures was generally less than in suspensions of *Pseudomonas saccharophila*, synthesis during growth in a glucose medium closely approaching the value for assimilation by resting cell suspensions, while with other substrates greater discrepancies were observed. With glucose as the substrate, 54% of the carbon was assimilated by the cells during growth while 60% assimilation was observed with resting suspensions. (It should be noted that the figures for assimilation by resting cells were based on carbon dioxide production rather than increased carbon content of the resting cells). With glucose, the ratio of assimilation during growth to assimilation by resting cells was 0.90, corresponding ratios for maltose being 0.83, sucrose 0.80, trehalose 0.84, lactate 0.72, pyruvate 0.60, and acetate 0.62. No values for residual carbon in the culture or suspension medium were reported.

Clifton (unpublished results) determined oxygen consumption and carbon dioxide production, during oxidative assimilation, and carbon contents of the cells and supernatant medium at the time a marked break in respiratory activity was observed. With *Prototheca zopfii* as the test agent and glycerol as the substrate, the oxygen consumption was approximately 27% of the theoretical for complete combustion and the R.Q. was 0.65 (theor., 0.86). Observed increases in carbon content of the cells utilizing glycerol above that observed in control (substrate-free) suspensions varied



from 0.232 to 0.372 mg. carbon when 0.432 mg. glycerol carbon had been utilized. According to the equation for glycerol utilization, as postulated by Barker (see Table II), five-sixths of the glycerol carbon is assimilated and on the basis of Barker's equation the assimilated carbon should amount to 0.36 mg. A closer consideration of Barker's results and of the oxygen and carbon dioxide values in these studies indicates that the postulated assimilation should probably be somewhat less than five-sixths (0.36 mg.) Transfer of *P. zopfii* from the Warburg vessels to the centrifuge combustion tubes is very difficult, as the organisms tend to be carried up the sides of the vessels and pipettes in films of water. Therefore the results of the carbon determinations are variable, but do indicate an actual assimilation of carbon. In one experiment in which least experimental difficulty was encountered, the increases in carbon amounted to 0.310 mg. in the cells and 0.032 in the supernatant, while 0.088 mg. was accounted for as carbon dioxide, a total recovery of 0.429 mg. from 0.432 mg. glycerol carbon. Thus it is apparent that assimilation by *P. zopfii* more closely approximates that postulated by equations based on manometric studies than does assimilation by yeast.

With *E. coli* (unpublished results) fewer experimental difficulties were encountered and the results tend to be more reliable. Typical results of studies on the assimilation of glycerol and of glucose in phosphate buffer at pH 7.2 and 37° C. are recorded in Table IV. The results of these studies suggested that the oxidation of glycerol by *E. coli* is more accurately represented by:



than by the equation postulated by Clifton and Logan (see p. 280). This equation predicts an oxygen consumption of 61% of the theoretical, carbon dioxide production of 58% of theory and an R.Q. of 0.78, the observed values being 60.1–62.0%, 58.2–60.0%, and 0.80, respectively. It is apparent that with glycerol as the substrate, the amount of carbon assimilation corresponds fairly closely to that predicted from the results of manometric studies, while with glucose as the substrate the difference is slightly greater, approximately three-fifths of the carbon postulated to be assimilated being found in the cells, while two-fifths is present in the medium in a form other than that of a reducing sugar. Formate appears to be oxidized completely to carbon dioxide and water by *E. coli*. In the presence of both glucose and formate the rate of oxygen consumption was greater than with either substrate singly, but the total amounts of oxygen consumed and of carbon dioxide produced were equal to the sums of the amounts in-

TABLE IV

OXIDATIVE ASSIMILATION OF GLYCEROL AND OF GLUCOSE BY *Escherichia coli* IN *M*/15 PHOSPHATE BUFFER OF pH 7.2 AT 37° C.

Oxidative assimilation of 0.432 mg. glycerol carbon. Postulated assimilation 0.162 mg. C.			
O <sub>2</sub> consumption, per cent theoretical.....	60.1	62.3	62.0
C in respiring cells, mg.....	1.812	1.822	1.834
C in control cells,* mg.....	1.680	1.680	1.680
Increase in cellular C, mg.....	0.132	0.142	0.154
C in CO <sub>2</sub> , mg.....	0.251	0.260	0.258
C recovered, mg.....	0.383	0.402	0.412
Oxidative assimilation of 0.70 mg. glucose carbon. Postulated assimilation, 0.233 mg. C.			
O <sub>2</sub> consumption, per cent theoretical.....	63.7	64.3	
C in respiring cells, mg.....	1.755	1.755	1.767
C in control cells,* mg.....	1.590	1.621	1.590
Increase in cellular C, mg.....	0.165	0.134	0.177
C in supernatant,* mg.....	0.110	0.110	0.095
C in CO <sub>2</sub> , mg.....	0.441	0.441	0.449
C recovered, mg.....	0.716	0.685	0.721
			0.690

\* Mg. C in controls identical in duplicate glycerol assimilation experiments, while variation observed in glucose experiments. Results of latter expressed on basis of different blank values. Mg. C in supernatant corrected for identical blank values.

volved in the oxidation of each substrate by itself. The same amount of carbon was assimilated from glucose as from glucose plus formate, thus indicating that formate carbon is not assimilated, although formate is readily oxidized.

Other studies concerned with the oxidative assimilation of substrates, or illustrating relations between respiration and synthesis, were reported by Fromageot and Safavi (32) with propionic acid bacteria. Hoover and Allison (43) and Burris and Wilson (14) with root nodule bacteria. Doudoroff (24) and Bernstein (9) with *Pseudomonas saccharophila*, van Niel and Cohen (86) with *Candida albicans*, McElroy (63) with luminous bacteria, and Foster (30, 31) with *Ps. riboflavina*. The results of these studies support the concept of oxidative and of fermentative assimilation and will be discussed in the next section.

#### IV. Influence of Poisons on Assimilation

With the results of Barker (5) and of Giesberger (38) in mind, Clifton (15) studied the possibility of bringing about a complete combustion of the substrate by blocking the assimilatory processes by means of suitable cell poisons. That indeed this blocking could be attained seemed to be suggested by the studies of Yamamoto (96), which showed that in growth ex-

periments the relation between synthesis and respiration could be altered by the addition of potassium cyanide, carbon monoxide, phenylurethan, or sodium fluoride to the medium. Also Clowes and Krah1 (18) demonstrated that the nitrophenols, and Krah1 and Clowes (50) that the dihalo- and trihalophenols stimulated oxygen consumption of eggs of the sea urchin (*Arbacia punctulata*) and at the same time inhibited cellular division of fertilized eggs; this effect might possibly be attributed to interference with assimilatory processes.

Other instances of selective poisoning of special metabolic processes are found in studies of Lundsgaard (61) and of McAnally and Smedley-Maclean (62). As is well known, Lundsgaard (61) showed that iodoacetate may fully inhibit fermentation while respiration is maintained at its normal intensity. Kluver and Hoogerheide (48) showed that higher concentrations of this poison inhibit respiration as well.

McAnally and Smedley-Maclean (62) made a study of the influence of sodium fluoride on glycogen storage by yeast during incubation in a glucose or maltose medium, both in the presence and absence of added phosphate. When yeast was incubated in a solution of glucose or maltose, the addition of even 0.01% sodium fluoride to the medium caused a marked inhibition of glycogen storage. If, however, 0.05% phosphate was also added to the glucose medium, the addition of 0.01% sodium fluoride did not diminish, but instead markedly increased the amount of glycogen stored; with further increase of the fluoride concentration to 0.03% the glycogen content steadily diminished.

Glycogen storage in a maltose-phosphate medium was more efficient than in pure maltose medium; the addition of 0.01% sodium fluoride to the maltose-phosphate caused no further increase of glycogen storage but sometimes a diminution. The addition of 0.01% sodium fluoride to the pure maltose medium, however, always produced a marked diminution of glycogen storage, while concentrations above 0.01% produced a diminished storage both in the presence and absence of phosphate.

In fermentations lasting 48 hours, addition of phosphate to glucose solutions increased the amount of sugar decomposed but produced no similar effect when added to maltose solutions. Upon the addition of fluoride to the media the amount of sugar which underwent decomposition was diminished. This effect was largely counteracted by the addition of phosphate.

Clifton (15) studied the influence of certain cell poisons on the oxidation of acetic and butyric acids by *Pseudomonas calco-acetica* in a phosphate buffer. The oxidation of acetate was characterized by a slight lag period, the rate of oxidation rapidly increasing to a relatively constant value which

was maintained until approximately two-thirds of the oxygen required for complete combustion had been consumed. At this time the rate of oxidation rapidly decreased and, by the time three-fourths of the oxygen required for complete combustion had been consumed, the rate of oxygen consumption had decreased to a level near that of the substrate-free control.

The effect of different concentrations of sodium fluoride, potassium cyanide, iodoacetate, sodium azide ( $\text{NaN}_3$ ), 2,4-dinitrophenol (DNP), and methylurethan on the oxidation of  $M/100$  acetate by *Ps. calco-acetica* was studied at pH 7.1. Concentrations of sodium fluoride as high as  $M/100$  had no appreciable effect on the rate or extent of oxidation of acetate. Potassium cyanide, in concentrations of  $M/800$  or higher, markedly inhibited the rate of oxidation, the degree of inhibition decreasing with time. A slight tendency for the oxidation to proceed nearer to completion was observed in the presence of cyanide.

Iodoacetate in concentrations greater than  $M/80,000$  decreased the rate of oxygen consumption below that observed in the presence of acetate alone. In the presence of  $M/10,000$  to  $M/70,000$  iodoacetate the total oxygen consumption was greater than in the presence of acetate alone, the rate of oxygen consumption remaining quite constant until approximately 90% of the theoretical amount of oxygen required for complete combustion had been consumed.

A behavior similar to that observed in the presence of iodoacetate was noted when sodium azide ( $M/600$  to  $M/1000$ ) or 2,4-dinitrophenol ( $M/4000$  to  $M/8000$ ) was added to the suspensions. Typical results of studies on the rate and extent of oxygen consumption during the oxidation of acetate by *Ps. calco-acetica* in the presence of these poisons are presented in Figures 2 and 3. It will be noted that the oxidation of acetate approached completion in the presence of suitable concentrations of azide or DNP. Similar results were observed during the oxidation of acetate by *E. coli* in the presence of these poisons. With either organism these poisons in the concentrations employed had little effect on the endogenous respiration; in general a slight reduction was observed. Low concentrations of DNP tended to stimulate to a slight extent the rate of oxygen consumption of *E. coli* in the presence of acetate. Addition of these poisons (unpublished results) to the respiring cells, after the break in rate of oxygen consumption was observed, had little or no effect on the rate of oxygen uptake. No evidence that DNP stimulated the exogenous respiration of *Ps. calco-acetica*, under the conditions of the experiments, was observed. In concentrations greater than  $M/8000$ , DNP slightly decreased the rate of respira-

tion and at the same time considerably decreased the amount of acetate assimilated by the bacteria.

Methylurethan in a concentration of  $M/10$  prevented assimilation during the oxidation of acetate in a manner similar to that described for the other poisons.

The oxidative assimilation of butyrate was of particular interest since the respiratory quotient during the stage of rapid oxidation was approximately 0.68, while the theoretical R.Q. for complete combustion is 0.8. The rate of oxygen consumption abruptly decreased to a level near that of

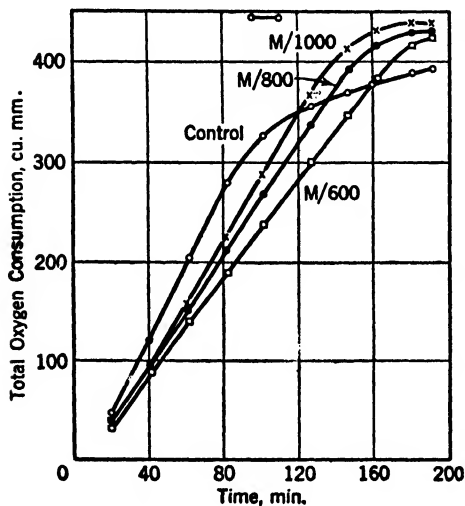


Fig. 2.—Influence of different concentrations of sodium azide on oxidation of acetate by *Pseudomonas calco-acetica* (15).

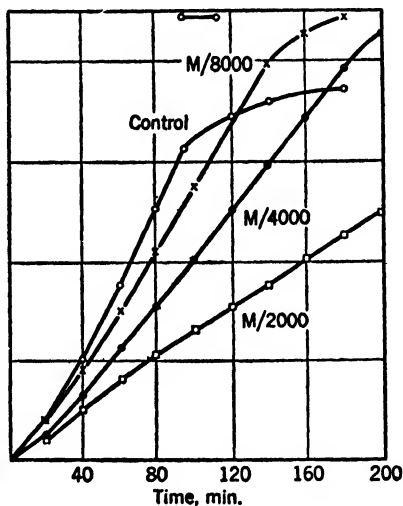


Fig. 3.—Influence of different concentrations of 2,4-dinitrophenol on oxidation of acetate by *Ps. calco-acetica* (15).

the control by the time three-fourths of the oxygen required for complete combustion had been consumed and the R.Q. increased to 0.94, a value characteristic of the respiration of the control suspension. However, in the presence of suitable concentrations of sodium azide or of DNP, the oxidation proceeded to completion at a quite constant rate and the R.Q. throughout was equal to the theoretical value 0.8. This furnishes more convincing proof that the agents inhibit synthesis to a greater extent than respiration, thus permitting the oxidation to proceed to completion.

That the processes of synthesis and respiration appear to be closely connected and influenced by the same agents was brought out by the use of

various concentrations of the poisons. Low concentrations of the poisons inhibit respiration slightly and simultaneously partially prevent synthesis. Critical concentrations of the inhibitory agent completely prevent synthesis and force the reaction in the direction of complete oxidation to carbon dioxide and water, the rate of oxygen consumption being somewhat further reduced. Higher concentrations of the poisons reduce the rate of respiration to a negligible value.

Clifton and Logan (17) extended the observations with *E. coli* and demonstrated that sodium azide and DNP in suitable concentrations tend to bring about complete oxidation of acetate, lactate, pyruvate, glycerol, fumarate, succinate, and glucose. However, these poisons tended to decrease the rate of oxidation of fumarate or succinate to a greater extent than that of the other substrates listed. The rate of oxidation of succinate by *E. coli* was inhibited almost 50% by  $M/16,000$  DNP although the oxidation did tend to approach completion; while concentrations of DNP as high as  $M/2000$  had little influence on the rate of oxidation of glycerol, this oxidation approaching completion in the presence of  $M/2000$  DNP.

Winzler and Baumberger (93) and Winzler (91) observed complete oxidation of acetate by yeast (determined on the basis of heat production or of oxygen consumption) in the presence of low concentrations of DNP, of sodium cyanide, or of sodium azide. These observations were extended (92) to include the anaerobic utilization of glucose by yeast. It was demonstrated that sodium azide in appropriate concentration completely prevented assimilation, practically 100% of the theoretical carbon dioxide production from a known amount of glucose being observed within 10 to 40 minutes. Sodium azide also appeared to inhibit ammonia uptake by yeast under anaerobic conditions (94).

Doudoroff (24) reported that a number of sugars as well as lactic and pyruvic acids were utilized by *Pseudomonas saccharophila*, apparently two-thirds of the substrate being assimilated by the cells. In the presence of DNP these substrates were oxidized to completion. However, pyruvic acid accumulated in the suspension medium during the course of oxidation of glucose in the presence of DNP and, being utilized at a slower rate than glucose, increased the time required for complete oxidation. Bernstein (9) demonstrated that DNP decreases the extent of assimilation with dicarboxylic acids, the inhibition of assimilation being less marked than with other substrates utilized by *Ps. saccharophila*. Foster (30, 31) observed complete oxidation of the substrate, riboflavin, by *Ps. riboflavina* in the presence of suitable concentrations of DNP.

Pickett and Clifton (68) studied the oxidative assimilation of several

substrates by different species of bacteria and the influence of DNP and of azide on assimilation. Their results are summarized in Table V.

TABLE V  
OXYGEN CONSUMPTION BY VARIOUS ORGANISMS (68)\*

Organism	Acetate	Pyruvate	Lactate	Glycerol	Glucose	Glucose + DNP		Glucose + NaN <sub>3</sub>	
						DNP concn.	%	NaN <sub>3</sub> concn.	%
<i>Staphylococcus albus</i>	121	84	65	72	66	M/3000	72	M/100	73
<i>Micrococcus luteus</i>	100	55	34	NU	57	.....	..	.....	..
<i>Aerobacter aerogenes</i>	105	77	56	50	63	M/2000	99	M/150	86
<i>Aerobacter cloacae</i>	95	69	73	47	56	M/2000	85	M/300	99
<i>Proteus vulgaris</i>	113	65	65	46	60	M/16,000	85	M/400	81
<i>Eberthella typhosa</i>	96	59	31	43	47	M/3000	72	M/100	73
<i>Salmonella schottmülleri</i>	84	52	64	37	50	M/2000	93	M/150	85
<i>Shigella dysenteriae</i>	NU	57	69	61	62	M/8000	79	.....	..
<i>Serratia marcescens</i>	..	74	74	67	68	M/4000	95	M/200	92
<i>Pseudomonas aeruginosa</i>	83	70	76	67	81	M/2000	98	M/400	115

\* Expressed as per cent of theoretical for complete oxidation, in M/200 substrate (M/400 for glucose) and M/15 phosphate buffer of pH 7 at 37° C. Gas phase, air. NU = substrate not utilized.

These observations indicate that the oxygen consumption by several species of bacteria during the oxidation of acetate may be greater than the theoretical amount required for complete oxidation. This apparent anomaly was observed not only with all substrates being oxidized by bacteria with a relatively high endogenous oxygen consumption, but also with substrates oxidized slowly by organisms having a low endogenous respiration. This phenomenon of oxygen consumption greater than theoretical was most apparent with *Bacillus subtilis* (unpublished results) with which the observed  $Q_{O_2}$  with added substrate was never greater than four times that of the control without added substrate. The results summarized in Table V together with unpublished results, also indicate that the effect of DNP or of azide on oxygen consumption in the presence of glucose varied not only between species of organism but also in a given species. This suggested that the poisons act upon more than one enzyme, or enzyme system, and that the blockade of assimilation might be the result of a number of actions and interactions.

Pickett and Clifton (66, 68) reported that the aerobic synthesis of carbohydrate by yeast was completely inhibited by M/1000 DNP. However, the oxygen uptake was not appreciably greater in the presence of this poison, the glucose postulated as assimilated in the absence of this poison being converted to carbon dioxide and alcohol in its presence (see Table

VI). Both oxygen consumption and synthesis were inhibited by sodium azide, the glucose in the presence of this poison being almost entirely fermented under aerobic conditions. Acetate, on the other hand (Winzler, 91), is completely oxidized to carbon dioxide and water in the presence of these poisons.

TABLE VI  
EFFECT OF SODIUM AZIDE AND OF DNP ON AEROBIC UTILIZATION OF GLUCOSE BY YEAST (66)\*

Poison	O <sub>2</sub> , $\mu$ l.	CO <sub>2</sub> , $\mu$ l.	R.Q.	Per cent of substrate		Total
				Oxidized	Fermented	
.....	466	481	1.03	34.7	3.1	37.8
<i>M</i> /1000 DNP	495	779	1.57	36.8	63.4	100.2
<i>M</i> /10,000 NaN <sub>3</sub>	543	691	1.27	40.4	30.3	70.7
<i>M</i> /2000 NaN <sub>3</sub>	104	520	5.00	7.7	85.3	93.0

\* Each Warburg vessel contained 0.1 cc. of *M*/10 glucose, 0.1 cc. of poison (or H<sub>2</sub>O) and 1.8 cc. of a *M*/15 phosphate buffer (pH 6.0) suspension of yeast.

As a confirmation of the studies with the Warburg technique, experiments were carried out on a semimacro scale, subsequent analyses being made for alcohol, glycerol, glucose, and of reducing sugar content of the yeast following acid hydrolysis. Typical results are presented in Table VII. They show that alcohol accounts for 30–40% of the glucose disap-

TABLE VII  
RECOVERY OF INITIAL AND END PRODUCTS FROM YEAST SUSPENSION (66)\*

Medium	Glucose only	Glucose + <i>M</i> /10,000 NaN <sub>3</sub>	
Carbon added as glucose, mg.	14.40	14.40	14.40
Carbon recovered, mg., as:			
Carbon dioxide	6.00	4.42	5.38
Alcohol	0.18	5.91	5.34
Glycerol	0.36	0.58	0.51
Glucose in medium	1.32	0.43	0.18
Synthesized carbohydrate	3.88	1.08	trace
Total	11.74	12.42	11.41
Per cent carbon recovered	81	86	79

\* Warburg experiment terminated as soon as rate of metabolism fell to endogenous level, and cells immediately separated from medium by centrifugation. 10 cc. yeast suspension in *M*/15 phosphate buffer at pH 6.0, glucose *M*/50, 37° C.; oxygen atmosphere.

pearing from the suspension medium in the presence of *M*/10,000 azide and at the same time synthesis of carbohydrate is markedly inhibited. (A similar decrease in the amount of assimilated matter on the basis of dry weights of the cells was also observed.) Pickett and Clifton (68) presented



additional data and a discussion of the influence of these poisons on the oxidative utilization of glucose by yeast. It is evident that the mode of action of these poisons is far from simple and that variations are observed not only between results obtained with different species, but also with the same species utilizing different substrates.

Clifton (unpublished results, see also Table IV) observed that the actual amounts of carbon assimilated may be considerably less than that postulated on the basis of manometric experiments. Furthermore, the addition of sodium azide or of DNP to the suspension medium resulted in a marked diminution in carbon assimilation from glucose or glycerol by *E. coli*. In typical experiments 0.20 mg. of carbon was assimilated from glucose, while assimilation was reduced to 0.08 and 0.04 mg. of carbon in the presence of DNP and of azide, respectively.

Burris and Wilson (14) observed increased oxygen consumption, but not oxidation to completion, during the oxidation of glucose by *Rhizobium trifolii* in the presence of DNP. This increase in the total amount of oxygen consumed was also evident when the poison was added after the break in rate of oxygen consumption and depletion of glucose in the medium was observed. This suggested that the poison did not inhibit assimilation but instead stimulated the oxidation of material assimilated during the period of rapid oxygen consumption. They also demonstrated that the addition of DNP to suspensions of *R. trifolii* or of *R. meliloti*, following preliminary incubation of the cells in glucose solution and washing by centrifugation, gave rise to both an increase in rate and total extent of oxygen consumption. DNP, in the concentration employed, depressed the respiration of control cells given no preliminary incubation in glucose solution. Thus, it appears that DNP does stimulate the oxidation of material assimilated from glucose by rhizobia.

Burris and Wilson also observed that glucose may disappear from the medium before the break in rate of oxygen consumption is observed. Strains of rhizobia also appeared to vary in their ability to oxidize and to assimilate glucose and in their response to DNP. Sodium azide, which is frequently more effective than DNP in preventing assimilation, was not as effective as DNP with rhizobia since azide strongly inhibits their respiration. These workers found that the delayed addition of DNP to suspensions of *E. coli* had little effect on rate and extent of oxygen consumption, while oxidation to completion was observed when the poison was added at the same time as the glucose.

McElroy (63), in a study of the effect of various narcotics on the extent of glucose oxidation by *Achromobacter fischeri*, observed that the oxygen

uptake at the point of glucose exhaustion was equivalent to 17% of the theoretical value for complete oxidation. Chloral hydrate and chloretone appeared to stimulate the respiratory utilization of cellular material assimilated during growth (not respiration alone), as approximately equal stimulation was observed with or without the addition of glucose to the suspension of washed bacteria. In the presence of chloral hydrate, but not of chloretone, there was, however, an increase in oxygen consumption of 8% of the theoretical after correction for stimulated endogenous respiration. The barbiturates and chloral hydrate exerted little effect if added after glucose had disappeared, whereas chloretone caused an immediate increase in rate of oxygen consumption. This behavior is similar to that reported for the effect of phthiocol (35) and of cyanide (27, 37) on *Chlorella*, these agents apparently stimulating the oxidation of internal material while inhibiting the oxidation of external substrate. DNP decreased rather than increased, the total oxygen consumption from a given amount of glucose. The action of DNP was attributed to an inhibition of oxidation of intermediate products of glucose oxidation.

Gaffron (35) has demonstrated that substances naturally occurring may inhibit respiration and synthesis in certain concentrations. Vitamin K or phthiocol in low concentrations stimulates respiration without inhibiting photosynthesis; in higher concentrations photosynthesis is inhibited without the stimulated respiration being appreciably effected, and in still higher concentrations respiration is inhibited. Thus, photosynthesis appears to be more sensitive than respiration, just as assimilation by microorganisms frequently appears to be more sensitive to certain poisons than is respiration.

Thus, it is apparent that the action of various poisons on the respiration of different species of microorganisms may vary with the nature of the substrate and of the organism. This latter variation has been applied by Snyder and Lichstein (74) in the development of a differential medium, the growth of common Gram-negative bacteria being inhibited to a much greater extent than that of Gram-positive forms by a concentration of 0.01% sodium azide in nutrient agar. Lichstein and Soule (56) pointed out that variations in sensitivity to the inhibitory action of sodium azide were observed within the Gram-positive and Gram-negative groups and that this agent appeared to inhibit aerobic respiration and growth to a greater extent than anaerobic activities.

The studies of Pickett and Clifton (68) and of Burris and Wilson (14) indicate that care must be exercised in interpreting the results of manometric experiments, but that in general certain poisons in appropriate con-

centrations do appear to inhibit assimilation, whether it be by blockade of the assimilatory reactions, diversion of substrate to secondary paths of breakdown, or by increased rate of oxidation of assimilated matter, thus possibly obscuring any concomitant assimilation. Extensive studies with "tagged" atoms would greatly facilitate the study of oxidative and fermentative assimilation as well as the influence of poisons on these reactions.

In general, the studies on the relation between respiration and assimilation suggest that there may be a stoichiometric rather than an energetic relationship between the anabolic and catabolic activities of the cell. Clifton and Logan (17) demonstrated that the assimilatory reactions appear to be functions of the chemical constitution of the substrate rather than of the available energy, since the same amount of carbon is assimilated from lactic as from pyruvic acid or from succinic as from fumaric acid, the free energy of oxidation of the first acid being greater than that of the second in each pair. Likewise, the heat or free energy of combustion of succinic acid is greater than that of lactic or pyruvic acid. Yet only one carbon atom is assimilated by *E. coli* from the four present in succinic acid as compared with one from the three-carbon compounds, lactic or pyruvic acids. Likewise, one carbon atom is assimilated per every four carbon atoms of acetate (or alcohol) disappearing from the suspension medium. The oxidative assimilation of two molecules of lactic or pyruvic acid gives rise to only two atoms of carbon assimilated, while three carbons are assimilated per mole of glucose utilized by *E. coli*, the total free energy decrease not being markedly different in these oxidations. Also, the oxidation of formate is accompanied by liberation of heat or energy; yet no assimilation from formate was observed either by the manometric technique or from microcarbon determinations on *E. coli* before and after formate oxidation.

One is thus led to consider the possibility (see also van Niel, 83) that catabolic processes may be of more importance in furnishing raw materials for assimilatory processes, which may then take place spontaneously rather than simply as a source of energy. Let us assume that a substrate is available for assimilatory purposes only after it has been broken down to an assimilable compound, *e. g.*, acetaldehyde. Then the amount of assimilation that could occur is a function of the maximum aldehyde that could be produced and utilized, rather than of the amount of energy liberated during the utilization of the substrate.

The results suggest that assimilation may arise from more than one fundamental "building block" such as acetaldehyde. Equivalent assimilation per four carbon atoms is observed with *E. coli* utilizing ethyl alcohol,

acetate, succinate, or fumarate, and the possibility exists that ethyl alcohol and acetate are oxidatively condensed to succinate and that oxidative assimilation is initiated during the course of succinate oxidation. Since a higher ratio of synthesis to carbon combusted is evident with lactate or pyruvate, it is possible that these compounds give rise to different intermediates and are not oxidized via succinic acid after a preliminary uptake of carbon dioxide. On the other hand, perhaps, too much emphasis should not be placed upon the results of manometric studies alone. Also, cell "mediators" may control the rate of synthesis, excess "building blocks" at times being oxidized or possibly excreted by the cell.

Different organisms may well use different intermediates or they may bring about the degradation of foodstuff molecules by different pathways. Also the equations postulate that a substance with the empirical composition of a carbohydrate is assimilated in the primary assimilation process, but the nature of this assimilation product is unknown. Evidence is accumulating that it need not always have as simple an empirical formula as  $\text{CH}_2\text{O}$ . Ruben *et al.* (71) (see also 28, 34, 44) have demonstrated that the carbon dioxide assimilated during photosynthesis gives rise to a substance with the empirical composition  $\text{CH}_2\text{O}$ ; yet the assimilated carbon appears to be fixed in a relatively complex molecule of high molecular weight which may serve as a reservoir of carbon for the synthetic processes of the cell. It is possible that the carbon assimilated during oxidation or fermentation may, initially, either form condensation products of the primary "building blocks" or may couple with complex molecules within the cell, the secondary reactions of synthesis proceeding from these reservoirs.

No attempt has been made in this discussion to postulate the role played by phosphate bonds or of carbon dioxide assimilation in the assimilatory processes. Undoubtedly, energy-rich phosphate linkages (see 46, 60) are involved in the degradatory, assimilatory, and synthetic processes, but no evidence has been obtained (Clifton, unpublished studies) that there is an appreciable accumulation of a primary assimilatory product rich in phosphate-bond energy since no appreciable phosphate uptake is observed in the course of oxidative assimilation. Energetic coupling during the synthetic processes in all probability involves phosphate-bond energy, the phosphate content of cells of *E. coli* apparently being sufficient to carry out these phosphorylations without uptake of inorganic phosphate from the medium.

Also the uptake of carbon dioxide may be involved in the assimilatory process, particularly with pyruvate or substrates which give rise to pyruvate on degradation (see 88).

It is to be remembered that the study of oxidative and fermentative assimilation is still in the exploratory state and, for the most part, the results are but suggestive of a general concept of chemosynthesis as a series of linked changes and interchanges between "building blocks" provided during the degradation of the substrate molecule. Many of these reactions could proceed spontaneously and from this viewpoint anabolism and catabolism are not energy-linked independent processes, but are chemically linked, stoichiometric processes, the free energy liberated as a result of cellular oxidations thus being a waste product of respiration along with carbon dioxide and water or the end products of anaerobic respiration.

## V. Assimilation of Carbon Dioxide

Wood and Werkman (95) presented the first experimental evidence that heterotrophic nonphotosynthetic bacteria assimilate carbon dioxide. The evidence for this assimilation has been reviewed by Werkman and Wood (88) and will not be considered in detail in this review. It is becoming apparent that the heterotrophic fixation of carbon dioxide may occur in a number of reactions, the best-established mechanism being that in which a four-carbon dicarboxylic acid is formed as a result of the condensation of carbon dioxide and a three-carbon compound, generally pyruvic acid.

The assimilation of carbon dioxide by photosynthetic bacteria has been discussed by van Niel (84) who pointed out that photosynthesis in general might be interpreted as a reduction of carbon dioxide. Evidence is accumulating that the autotrophic bacteria also fix carbon dioxide by means of a reductive mechanism, similar to or identical with that invoked in photosynthesis. The fixation of carbon dioxide by photosynthetic forms may well be a "dark" reaction and the main difference between photosynthetic autotrophic and chemosynthetic autotrophic carbon dioxide assimilation may lie in the nature of the energy source for the assimilation.

Vogler and Umbreit (87) reported that the oxidation of sulfur and fixation of carbon dioxide by the autotrophic bacterium, *Thiobacillus thiooxidans*, is coupled to transfer of phosphate from the medium to the cells. When *T. thiooxidans* was allowed to oxidize sulfur in the absence of carbon dioxide, the concentration of phosphate in the medium decreased. The inorganic phosphate disappearing from the medium was accounted for as organic phosphate esters, later identified by LePage and Umbreit (53) as phosphorylated carbohydrate esters characteristic of yeast and muscle metabolism with the exception (54) that adenosine-3-triphosphate was observed rather than adenosine-5-triphosphate. The phosphate appeared to

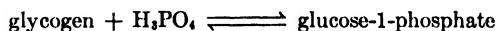
be stored in the esters as energy-rich phosphate bonds. The energy thus stored in the phosphate bonds was found to be available at a later time in the complete absence of sulfur oxidation for the fixation of carbon dioxide. This fixation was accompanied by a release of inorganic phosphate to the medium. This phosphate turnover is not readily observed in cells actively oxidizing sulfur and assimilating carbon dioxide, because phosphate is released during carbon dioxide fixation as rapidly as it is esterified during the concomitant sulfur oxidation.

It is of interest to note that sulfur oxidation could continue for hours in the absence of carbon dioxide, the phosphate energy bonds formed probably being released by cell phosphatases since the cells are capable of storing only a limited amount of energy-rich material. Likewise, formate can be oxidized by *E. coli* at a steady rate for hours; yet no detectable assimilation occurs. Either sufficient energy is not available for assimilation, or more probably no intermediate metabolite is formed which can be utilized in an assimilatory reaction.

The studies with *T. thiooxidans* as well as with photosynthetic organisms also suggest that carbon dioxide fixation leads to the formation of one or a limited number of organic substances from which the manifold components of the cells arise by a series of secondary transformations of the primary assimilation product (s). Such a concept is obviously in agreement with that postulated for heterotrophic organisms and points to a unity of action throughout nature.

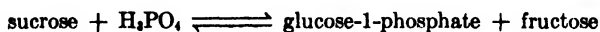
## VI. Polysaccharide Synthesis

The mechanism for the synthesis of glycogen or starch by animal and plant tissues became evident with the discovery of the reversibility of phosphorolysis. Cori, Schmidt, and Cori (22) demonstrated that the phosphorolysis of glycogen is a reversible reaction. In this reaction which may be pictured as:



the enzyme phosphorylase, with adenylic acid as a coenzyme, brings about glycogenolysis or glycogen synthesis. The synthesis of glycogen was shown to occur *in vitro* in the presence of the appropriate enzyme system.

More recently, Doudoroff, Kaplan, and Hassid (26) have demonstrated the synthesis of sucrose from glucose-1-phosphate and fructose by a dry preparation of *Pseudomonas saccharophila*. This reaction may be represented as:



This enzymic phosphorolysis of sucrose is a reversible reaction, occurs without concomitant oxidations or fermentations, and appears to compete in the enzyme preparation with the hydrolytic splitting of sucrose, an irreversible reaction. Thus, in the presence of the necessary enzymes and coenzymes and with suitable concentrations of reactants, the spontaneous synthesis of polysaccharides is possible. Kagan, Latker, and Zfasman (45) presented evidence for the phosphorolysis of sucrose with the production of glucose-1-phosphate by suspensions of *Leuconostoc mesenteroides*. Doudoroff, Hassid, and Barker (25) reported the enzymic synthesis of two new disaccharides, apparently glucosidosorboside and glucosidoketoxylside being formed upon the addition of glucose-1-phosphate and *l*-sorboside or *d*-ketoxylside, respectively. Leibowitz and Hestrin (52) in a review on the fermentation of oligosaccharides have pointed out that polysaccharide synthesis may occur without phosphorolysis. A number of bacteria ferment sucrose with the degradation of one-half of the sucrose molecule into smaller parts, the other half, glucose or fructose, being polymerized without phosphorylation with the formation, respectively, of a dextran or a levan.

## VII. Miscellaneous Syntheses

Among the typically catabolic processes we find concurrent syntheses such as those of dextran and levan. Also there have been numerous reports of the fermentative formation of compounds more complex than the original substrate. For example, Barker (6) has demonstrated the production of large amounts of caproic acid together with butyric acid during the methane fermentation of ethyl alcohol. Likewise, Silverman and Werkman (72) have described the preparation of an active enzyme system from *Aerobacter aerogenes*, catalyzing the dissimilation of pyruvic acid to carbon dioxide and acetylmethylcarbinol. Here again is an example of a spontaneously occurring enzymic synthesis of a compound containing more carbon atoms per molecule than are present in the substrate molecule. Isolated examples of this nature suggest that condensations may play an important role in syntheses, provided the substrate or its degradation products provide the necessary building blocks. Also the spontaneous synthesis of certain amino acids from their keto acid analogues and ammonia is a well-established fact and will be discussed in the next section.

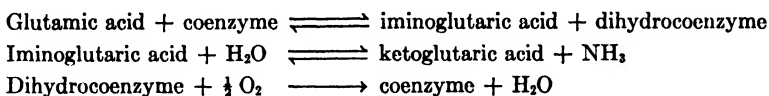
## VIII. Assimilation of Nitrogen

A limited number of microorganisms assimilate nitrogen itself, but the majority require combined nitrogen in the form of nitrates, ammonia, or

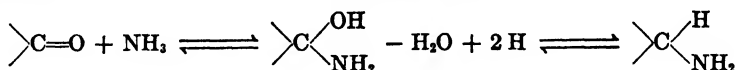
organic nitrogenous compounds, particularly the amino acids. Bacteria, for example, have been roughly classified on the basis of their nitrogenous requirements (see 49) and various intergradations are observed from the nitrogen fixers to the most exacting parasites.

The more recent studies on biochemical nitrogen fixation have been reviewed by Burk and Burris (13). The two main theories concerning the assimilation of nitrogen suggest that ammonia or hydroxylamine (or oxime) are the important intermediates in the transformation of nitrogen into cellular nitrogenous matter. Hydroxylamine, as an intermediate, appears to be involved in the utilization of nitrogen but may not be the initial fixation product. It could react with oxalacetate to yield oximino-succinate, which on reduction is converted to aspartic acid. However, much work remains to be done before a completely satisfactory scheme of nitrogen utilization can be realized.

It is generally assumed that the assimilation of ammonia may occur by means of reversible deamination reactions. Quastel and Woolf (69) (see also 21) demonstrated that washed suspensions of *Escherichia coli* deaminate aspartic acid with the production of fumaric acid and ammonia. When these suspensions were incubated in the presence of antiseptics, such as toluene, together with ammonia and fumaric acid, aspartic acid was formed, thus indicating the reversibility of the deamination reaction. Adler *et al.* (1-3) demonstrated the reversibility of the enzymic (including yeast and bacterial) decomposition of glutamic acid, the reaction being pictured as:



Oxalacetic acid may also react with ammonia with the resultant synthesis of aspartic acid. Lipmann (60) has suggested that ammonia may be introduced through addition on a carbonyl double bond (possibly on  $\text{—C=C—}$  also, as mentioned above for the synthesis of glutamic and aspartic acid) in a manner similar to the addition of phosphate in the formation of acyl phosphate. The reaction can be represented as:

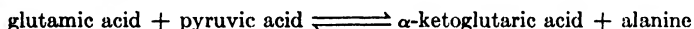


Virtanen and Laine (see Burk and Burris, 13) have postulated a similar type of reaction, involving the addition of hydroxylamine to oxalacetic acid with formation of aspartic acid, in the symbiotic fixation of nitrogen.



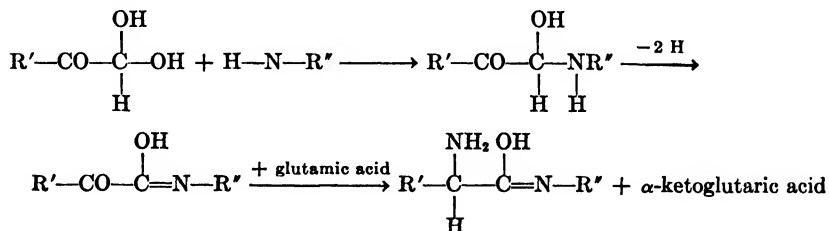
Numerous species of bacteria can grow in culture media with ammonia as the sole source of nitrogen. This suggests that the primary assimilation of ammonia must occur in reactions such as those just mentioned. With many bacteria the addition of amino acids to the culture medium increases the rate of growth, and this can be interpreted in two ways. Either the amino acid is employed directly, growth being retarded by a slow rate of synthesis of the amino acid, or the amino acid on dissimilation yields building blocks readily utilized by the cell. Possibly both mechanisms are involved. With other species the synthesis of a particular amino acid, *e. g.*, tryptophan (see 49), retards growth or the cells may be unable to synthesize tryptophan and growth therefore would not be evident. Still other species require two or more preformed amino acids for growth, thus suggesting a gradual loss of synthetic ability, generally observed as the organism becomes more parasitic.

Once amino acids have been synthesized, or are added to the medium, other amino acids may be formed by transamination reactions originally observed by Braunstein and Kritzmann (12) with animal tissues. A typical transamination reaction (see reviews by Cohen (19), Herbst (42), and Gale (36)) may be represented as:



a reaction in which an amino group is transferred from an  $\alpha$ -amino to an  $\alpha$ -keto acid or vice versa. Adler *et al.* (2, 3) have reported transamination with yeast extracts, *E. coli*, and lactic acid bacteria. Lichstein and Cohen (55) have also demonstrated transamination in several species of bacteria, the rate of transamination in some instances being greater than that observed with animal tissues.

Linderstrøm-Lang (58) has suggested that transamination may be involved in the synthesis of proteins, combining oxidation and transamination in a hypothetical scheme as follows:



It is quite possible that a phosphorylated aldehyde may be involved in the initial reaction instead of the hydrated  $\alpha$ -keto aldehyde.

Not only transamination, but also transaminidation, may be involved in the synthesis of nitrogenous matter. While the reviewer has not seen any studies on this reaction with microorganisms, it has been demonstrated by Borsook and Dubnoff (11) with kidney tissue. The amidine group,  $\text{H}_2\text{N}$

$\text{>C=NH}$ , in arginine may be transferred to glycine with the formation of guanidinoacetic acid and ornithine. The guanidinoacetic acid may in turn serve as a precursor for the formation of creatine by transmethylation from methionine.

As Lipmann (60) has pointed out, there are group donors and acceptors for acetyl-, phosphate-, methyl-, amino-, and amidino- groups and that transfers of these groups may be involved to a considerable extent in cellular synthesis. Further development of the group-transfer and group-potential concepts should markedly increase our knowledge of the synthetic processes.

Studies on growth factors for microorganisms yield results of importance to an understanding of the assimilatory processes. Knight (49) pointed out that specific growth factor requirements may be the result of the inability of an organism to synthesize a particular factor, or a component of that factor. For example, thiamin may be a specific growth factor because of an inability of the cell to synthesize the thiazole or pyrimidine components, to combine these components with the formation of thiamin, or because of the loss of several of these abilities. In some instances it is sufficient to supply one component of thiamin, the cells being able to synthesize the second component and to bring about the union of the synthesized and the preformed components.

Of particular interest are the recent studies on the biosynthesis of amino acids by fungi (reviewed by Tatum, 81). Most fungi can synthesize their amino acids from ammonia or nitrates. However, the growth rate is often increased by the addition of amino acids which suggests that the rate of amino acid synthesis may be a limiting factor.

Beadle and Tatum (7) developed a procedure for the study of biosyntheses by the ascomycete *Neurospora crassa* based on the assumption that irradiation of spores may induce mutation in genes concerned with the control of specific chemical reactions. If an organism derived from a normal spore must be able to carry out a specific reaction to maintain growth on a given medium, an induced mutant unable to carry out this reaction will not grow. Such a mutant will grow, however, if the essential product of the blocked reaction is supplied in the culture medium, provided the

substance can gain entrance into and be utilized in the chain of events leading to growth. Strains were obtained, following x-ray treatment, characterized by their inability to synthesize either vitamin B<sub>6</sub>, the thiazole half of thiamin, or *p*-aminobenzoic acid.

Later strains were obtained which specifically required an essential amino acid for growth, the genetic block being in a reaction specific for the synthesis of that particular amino acid. Blockade of the synthesis of the simpler or nonessential amino acids appears to be rare or improbable since their synthesis probably involves a reaction path or paths common to the synthesis of other intermediates. Such a mutant would be unable to multiply or even remain alive.

One mutant strain of *N. crassa* (10) was found to require the two amino acids isoleucine and valine for growth in the basal medium. Optimum growth was observed with a ratio of 7 to 8 parts of valine to 3 to 2 parts of isoleucine. An inadequate supply of valine could be supplemented by the addition of leucine. Growth was observed if the keto acid analogue of valine and isoleucine itself, or vice versa, was added to the basal medium. Growth was not observed when the keto acid analogues were substituted for both amino acids. The synthesis of isoleucine and valine appears to involve a common precursor or reaction, the nature of which was not determinable. Regnery (70) reported studies on a leucineless mutant strain of *N. crassa* obtained on ultraviolet irradiation. This strain grew on the basal medium following the addition of leucine or its keto analogue,  $\alpha$ -ketoisopropic acid.

Srb and Horowitz (76) demonstrated that the biosynthesis of arginine by *N. crassa* apparently involves an ornithine cycle (51) similar to that of mammalian liver. Seven genetically different strains were studied, each of which was unable to carry out certain steps in the synthesis of arginine. Synthesis of arginine appears to involve the order  $\rightarrow$  ornithine  $\rightarrow$  citrulline  $\rightarrow$  arginine. One strain was unable to convert citrulline to arginine, two were unable to convert ornithine to citrulline, and four could not synthesize ornithine from sucrose and ammonia. Consideration of the genetic differences between these strains suggested that there are two steps involved in the conversion of ornithine to citrulline and at least four steps in the synthesis of ornithine. Of interest at this point is the observation by Doermann (23) that arginine is inhibitory to the growth of a lysineless mutant of *N. crassa*.

The biosynthesis of tryptophan by *N. crassa* has been demonstrated by Tatum and Bonner (82) to involve a condensation of serine and indole. Evidence was also presented suggesting that the biosynthesis of tryptophan

by *E. coli* or the production of indole by this organism involves a similar condensation or reversal of the reaction, respectively. Anthranilic acid was found to be an intermediate in indole formation, one mutant strain being unable to form anthranilic acid but able to convert it to indole, while a second strain was blocked between anthranilic acid and indole.

Gray and Tatum (40) isolated mutants of *E. coli* and *Acetobacter melanogenum* following x-ray irradiation which lacked the capacity of carrying out specific biochemical reactions characteristic of the parent cultures. One mutant strain of *E. coli* was characterized by its inability to synthesize biotin, the other by requiring threonine. Four strains of *A. melanogenum* were differentiated, one requiring serine or glycine, a second adenine or adenosine, a third glycine, and a fourth showed improved growth upon the addition of leucine. Thus it is becoming apparent that microbial syntheses and assimilations are enzymically controlled step reactions, the enzymic pattern of the cell in turn being controlled by specific genes. For further details and for similar types of reactions in other fungi and higher cells or tissues the reader is referred to the review by Tatum (81).

Thus, it is apparent that the loss or alteration of one or more genes may markedly influence the synthetic ability of a cell and make it more dependent on preformed building materials. In the case of normal cells of *N. crassa*, abundant growth (synthesis and assimilation) is observed in an inorganic medium plus sucrose and biotin. In the mutants, certain synthetic abilities may be lost and the study of a considerable number of these mutants may give rise to a much better understanding of intermediate syntheses and assimilations. However, in the normal cell and to a great extent in the mutants, synthesis may well arise from one or a limited number of primary building blocks assimilated during the course of the dissimilation of sucrose.

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# CHEMICAL CHANGES IN THE HARVESTED TOBACCO LEAF

## Part I. Chemical and Enzymic Conversions during the Curing Process\*

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*"By Hercules! I do hold it and will affirm it,  
before any prince in Europe, to be the most  
sovereign and precious weed that ever the  
earth tendered to the use of man."*

BEN JONSON'S *Every Man in His Humor*, 1598

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\* It was the wish of the editor and the intention of the writer to present a complete survey of chemical changes in the tobacco leaf, including the two main phases of its processing—curing and fermentation. The material, however, proved to be so extensive that it was decided to publish as Part I a review only of those conversions which result from curing. A second report, scheduled to appear in a later volume of *Advances in Enzymology*, will deal with the changes during fermentation.



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## I. Introduction

Our knowledge of the chemical changes which occur during the processing of tobacco, from the freshly harvested plant to the "finished" tobacco ready to go into the product for smoking, is still very incomplete. Compared with other processes, such as the alcoholic fermentation which starts from vegetable materials rich in carbohydrates, the chemical reactions within the leaf tissue of tobacco present a complicated picture, of which only a few fragments have been explored thoroughly.

Various factors are responsible for this situation. Unlike the enzymic production of alcohol and other processes in which specific substances are obtained by the fermentation of vegetable matter, the chemical reactions in the tobacco leaf are manifold in nature. The only firmly established empirical fact is that when harvested tobacco leaves are properly treated by a number of successive processes, the result is a far superior smoking material to the untreated, merely dried tobacco leaves.

Although it has been shown that certain chemical changes occur in the course of this treatment, it has not yet been proved that these particular changes are the decisive ones for the improvement of the smoking properties. Additional, still undiscovered, reactions within the leaf tissue may exist which are of greater importance than the transformations found and studied so far. Another, and rather serious, complication is the wide variety of the tobacco types grown in various localities under divergent conditions and also the different kinds of treatment applied to the harvested leaves, depending upon the special product desired. An entire volume could be filled with a description of the various processes known as drying, curing, sweating, redrying, aging, resweating, and fermentation which have been developed for the manufacture of cigar, cigarette, and pipe tobaccos. Each of these operations has been adjusted to the special type and strain of tobacco used as the raw material, and is further modified according to the nature of the crop of a given "vintage." In view of the divergence between the different, empirically developed methods of processing tobacco, it has been assumed, and also proved, that these different processes are not merely slight variations of one and the same basic sched-

ule. On the contrary, in many cases the treatment applied to a given type of tobacco causes chemical changes which are opposite to those caused by the individual treatment of a second type of tobacco during its customary development to the finished product. It is, therefore, wrong and misleading to consider the chemical transformations which result from the processing of tobacco as identical or similar for the whole gamut of finished products. A study of the literature reveals that this misconception has often occurred, and that erroneous conclusions were reached as a result of it.

The complex and varied nature of the reactions during the processing of tobacco leads to a corresponding uncertainty regarding the mechanism of these changes. Despite the many arguments in favor of a purely chemical catalytic, a purely enzymic, or a purely bacteriologic nature of the transformations in the tobacco leaf during curing and sweating, no argument is yet completely convincing as to any one of these possibilities. Probably, the changes occurring during curing and fermentation are the result of all three processes. It will be necessary to obtain complete quantitative data on the chemical reactions in a few selected types of tobacco during standardized treatments in order to reach definite conclusions as to the mechanisms of the changes, and in order to decide whether some of the bacterial actions, for example, are indispensable components of the process, or merely accidental in nature.

In view of this situation, enzymic actions are not claimed, in this study, to be the predominant type of chemical reactions in the tobacco leaf, even though the trade term, "fermentation," of a part of its treatment may give the impression that this process can be exclusively ascribed to enzymic actions.

## II. The Green Tobacco Leaf: Its Components and Their Conversions

A short description of the composition and metabolism of the green tobacco plant will help one to understand the chemical processes during curing and sweating, not only because it presents the chemical character of the original material but also because the transformations in the living plants may provide the clue to some of the "post mortem" changes, or at least to the initial trend of these changes in the leaves.

### 1. Tobacco Types

About 42 species are known of the genus *Nicotiana* which, in its turn, belongs to the family *Solanaceae*. Of these 42 species, only two are being grown for use as smoking and chewing tobaccos and snuff, namely, *N. tabacum* L. and *N. rustica* L. (makhorka). The former predominates by far.

In this country, *N. rustica* is grown on a very small scale, as a source of nicotine only, but in a few countries, especially in Russia, it is also grown for smoking tobacco. Extensive cultivation of tobacco was first started by the Spaniards in their American colonies around 1530, and somewhat later (around 1600) the British began to raise it in their colonies in North America (100). Between 1580 and 1600 the plant was introduced into the countries of Europe, into China, Japan, South Africa, and many other countries. The Indians of North America grew mainly *N. rustica*, but the English settlers soon replaced it by *N. tabacum* (which probably originated in Brazil and other sections of Central and South America). Many special strains of *N. tabacum* have been developed during the three and a half centuries of tobacco cultivation. In the United States alone 26 types are officially classified as different varieties, and each of these varieties is grown locally in various subvarieties (77). Similar conditions prevail in other countries.

The specific characteristics of the various types can only in part be ascribed to the factor of inheritance. Mainly, they are the result of environmental conditions such as soil, climate, and practices of cultivation. This point has been repeatedly proved by the failure to transfer successfully a given type to another locality. As a rule, shortly after such a transfer the characteristics of the plant change to a remarkable extent. The susceptibility of the tobacco plant to soil and weather conditions is also demonstrated by the marked differences in plants of the same type which in one season have grown on plots of slightly different soil or fertilization, or grown on the same plot, in different years. The particularly high susceptibility of tobacco to environmental conditions is probably due to the fact that the modern cultivation of tobacco is close to the ceiling of efficiency. Present methods produce crops which grow very rapidly and yield maximum weight per acre because of the large amounts of fertilizer used. Obviously any slight disturbance of such a highly developed system will cause the plants to deviate from the ideal type which is obtained under favorable conditions.

## 2. Main Components of the Green Tobacco Leaf

In spite of this variability, certain features of the chemical composition of the tobacco plant appear so regularly that they add up to a typical, fairly constant, picture. Since leaves are the raw material of curing and fermentation processes, our survey deals with the composition of the tobacco leaf only, and not with the stalk or root of the plant. The composition of the leaf varies according to its age and position on the stalk. The distribution of substances within each leaf is not homogeneous, but these finer details are disregarded in order to obtain a general picture. Table I contains average percentage values of different classes of constituents in freshly harvested leaves of mature tobacco plants before drying, the data being obtained by compiling a great number of individual analyses as re-

corded in the literature (6, 7, 25, 32, 40, 52, 81, 104, 113, 119, 123, 131, 201, 238, 240, 242, 250, 258, 262, 305, 321, 335).

TABLE I  
AVERAGE COMPOSITION OF FRESHLY HARVESTED TOBACCO LEAVES

Type of compound	Per cent of dry weight of leaves	
	Cigar tobacco	Cigarette tobacco
<i>Static Group</i>	45.0	42.5
Ash (inorg. cations and anions)	14.0	12.0
Crude fiber (cellulose and lignin)	9.5	10.0
Pentosans	3.0	2.0
Pectins	7.0	7.0
Ether-sol. compds. (vol. oils, resins, waxes, paraffins)	7.0	7.5
Tannins (polyphenols, phenolic acids)	2.5	2.0
Oxalic acid	2.0	2.0
<i>Nitrogen Group</i>	24.0	15.5
<i>Total Nitrogen</i>	4.1	2.7
Proteins	See Table II	See Table II
Soluble N compds. (amino acids, amides, ammonia compds., nitrates, alkaloids, unidentified compds.)	See Table II	See Table II
<i>Dynamic Group</i>	31.0	42.0
Carbohydrates (poly- and monosaccharides, starch, dextrin)	3.0	23.0
Ether-sol. org. acids (citric, malic, and unidentified acids)	11.0	11.0
Unidentified compds.	17.0	8.0
<i>Total</i>	100.0	100.0

**The Static Group.**—As pointed out above, the average values listed in Table I can change by large amounts. Even for leaves of the same tobacco type, the variations can be as much as 40% and more, depending upon their age and position on the stalk, and weather, soil, and fertilization. It is significant, however, that some of the components are much less variable than others, an indication that the less variable substances are relatively constant constituents, more inert toward changes in growing conditions than the remainder. This is particularly true of the components of the leaf framework and cell walls, such as the crude fiber, pentosans, and pectins. The relatively inert inorganic substances (ash), and the ether-soluble compounds (resins, fats) also belong to this group, the last-named being the least stable. Almost all the substances listed in the Static Group are accounted for by analytical determination. At least 95% of the total inorganic substances in the ash of leaf samples can be identified, analytically, in the form of calcium, potassium, sodium, magnesium,

iron and aluminum, manganese, phosphorus, sulfur, silicon, and chlorine. Each of the organic substances in this group—the crude fiber, pectins, pentosans, tannins, oxalic acid, and ether-soluble resins and waxes—represents a definite class of compounds, with common chemical characteristics and reactivities. A complete separation into its individual members has not yet been carried out for any of these classes. Nevertheless, they are well enough defined and detectable by analytical methods to make an approximate survey of the composition of the leaf tissues possible.

**The Nitrogen Group.**—A number of authors have studied the nitrogen compounds contained in the leaves of various tobacco types. Most of the components of this group can be determined as individual compounds, or at least as individual groups of related compounds, falling into two main divisions: insoluble nitrogen, mainly proteins, and soluble nitrogen, mainly ammonia, amino compounds, nitrates, amides, and alkaloids of the nicotine type. About 85 to 95% of the total nitrogen compounds have been identified by analysis, as belonging to the one or to the other of the classes of nitrogen compounds just mentioned, the remainder being listed as unidentified nitrogen. Table I gives average percentage values of the total nitrogenous compounds for mature green leaves of typical cigar and cigarette tobaccos. The values given for total nitrogen (in italics) are more accurate than those for the total nitrogenous compounds.

The values for total nitrogenous compounds can be derived approximately from the total nitrogen by multiplying the latter by 5.8.<sup>\*</sup> This factor is the average of values calculated from several analyses in which the individual nitrogenous compounds were determined separately, and the total of their weights compared with that of the total nitrogen.

The total nitrogen varies considerably in different kinds of green tobacco leaves. Like the Static Group, it, too, depends on growing conditions, such as soil, amount and kind of fertilizer, weather, etc. Mature leaves of field-grown tobaccos show total nitrogen values ranging from about 1.3 to 6% (expressed as percentage of dry weight). Multiplication by 5.8 (the conversion factor) shows that the nitrogen compounds in the leaves of these different tobacco types vary from about 7.5 to 34.0%. The influence of the weather is generally toward a higher nitrogen content in plants grown in dry seasons.

On the average, leaves of cigarette tobaccos contain a considerably smaller amount of nitrogenous compounds than those of cigar tobaccos (Table I). The reason, apparently, is not only a basic difference between the two types of tobacco but also the fact that the leaves are harvested at different stages of maturity. As a rule, cigar tobacco plants are either cut whole, or the leaves are picked at full maturity, when they are at the peak of vitality. The leaves of most cigarette tobaccos, however, are primed when

overripe, when their vitality is on the decrease. In this state nitrogenous compounds have begun to migrate from the leaves into other parts of the plant (see page 337). The smaller proportion of nitrogenous compounds in cigarette tobaccos is also partly the result of the smaller amounts of nitrogenous fertilizers used in their cultivation, as compared with cigar tobaccos.

Table II lists average values of total nitrogen content of the green leaves of cigar and cigarette tobaccos and of certain principal classes of nitrogenous compounds. On the whole, the literature records a relatively higher

TABLE II

DISTRIBUTION OF NITROGEN COMPOUNDS IN FRESHLY HARVESTED TOBACCO LEAVES\*

Fraction	Cigar tobacco			Cigarette tobacco		
	Nitrogen content in per cent of		Nitrogenous compounds in per cent of dry wt.	Nitrogen content in per cent of		Nitrogenous compounds in per cent of dry wt.
	Total N	Dry wt.		Total N	Dry wt.	
Total nitrogen	100	4.1	24.0	100	2.7	15.5
Protein nitrogen	70	2.87	17.3	75	2.03	12.2
Soluble nitrogen	30	1.23	6.7	25	0.67	3.3
Amino	5	0.205	1.2	2	0.054	0.3
Ammonia plus amide	1	0.041	0.3	1	0.027	0.1
Nitrate	6	0.246	1.0	7	0.189	0.7
Alkaloid	12	0.492	3.0	10	0.270	1.3
Unidentified	6	0.246	1.2	5	0.135	0.9

\* After curing and fermentation, some of the differences between cigar and cigarette tobaccos disappear. The alkaloid content of well-fermented leaves of both tobacco types, is about 0.4 to 1.0%. However, cigar tobacco remains richer in total nitrogen.

proportion of proteins in the nitrogen fraction, for fresh leaves of cigarette tobacco than for those of cigar tobaccos. It is not yet completely clear whether the characteristic distribution of the nitrogenous substances actually differs in the intact leaves, or whether the difference mentioned is the result of very rapid autodigestion, up to and during analysis of the proteins to soluble compounds in the more vital cigar tobacco leaves.

The proportion of soluble nitrogen compounds is appreciably higher in the leaves of plants grown in dry seasons, probably because at such times the plants use part of their protein to keep up respiration. This is unnecessary when, as in normal seasons, the leaf tissue contains enough water and sufficient carbohydrates. In addition, the soluble nitrogen compounds, which normally are transported from the leaves to developing plant organs, accumulate in the leaves of the plants stunted by dry weather.

The amounts of nitrogenous compounds initially present in green tobacco leaves vary by more than 100% between different types of tobacco.

The change in total amount of nitrogen compounds throughout the drying, curing, and fermentation of any one type of tobacco leaf, however, is not very large, the decrease as a rule being no more than about 5 to 25% of the initial value. But *within* the groups of nitrogen compounds radical and significant shifts occur which result from transformations of one kind of nitrogenous compound into another, especially in the form of digestion of proteins to soluble nitrogen compounds (see Table III). These internal changes, which take place as a part of the nitrogen metabolism in the living green plant, are also an important part of the reactions during curing and fermentation (see page 337).

**The Dynamic Group.**—The least stable and least explored group of substances listed in Table I is the Dynamic Group. In addition to the carbohydrates, the group includes: (1) the ether-soluble organic acids, which are citric and malic acids (oxalic acid is included in the Static Group because it is relatively stable), and a relatively small proportion of unidentified acids; and (2) the remainder of analytically undetermined organic substances contained in the leaf tissue. This remainder is a major component of the group amounting to about 7 to 18% of the dry weight. It is probable that all these substances participate in the cycle of oxidative reactions which lead from the carbohydrates, through various intermediate stages, to carbon dioxide and water as final oxidation products.

The green leaves of the various tobacco types exhibit as large variations in the amounts of substances of the Dynamic Group as in their nitrogen contents. Both variations counterbalance each other, in the sense that tobacco types with small quantities of nitrogenous compounds generally contain large amounts of Dynamic Group substances, and vice versa. Hence, the sum of the compounds of both groups is fairly constant (54 to 60% of the dry weight) even for widely different tobacco types. The ratio, however, of nitrogenous to dynamic compounds varies from about 10/50 to 40/20, with 14/45 approximately the average for normal cigarette tobacco and 27/33 for normal cigar tobacco.

Determination of the compounds of the Dynamic Group and investigation of their chemical changes are difficult mainly for two reasons. The first is that, as a result of high reactivity and lability, many compounds of this group are rapidly transformed and lost from the leaf tissues in the form of volatile oxidation products. Probably, these losses start very shortly after the leaves are detached from the stalk and continue during drying, curing, and fermentation. The magnitude and speed of the weight losses depend on the type and crop of tobacco, stage of maturity, and conditions of storage and treatment.

Selection of the conditions which either favor or limit the chemical losses of these substances is a significant feature of the various types of treatment applied to to-

bacco. The practical loss in total weight during handling includes, in addition to the chemical losses, losses due to spoilage, mechanical breakage of leaf fragments, removal of the middle rib of the leaves, and loss of moisture from the end of curing to the end of treatment. The total loss for flue-cured cigarette tobacco can amount to as much as 36%, for air-cured cigar tobacco to as much as 42% of the weight after curing (77, 158).

Average values for the chemical losses of air-cured cigar and cigarette tobaccos and flue-cured cigarette tobacco are given in Tables IV, V, and XI. The fact that the rapidity of the decrease in weight varies with tobacco type and with different crops of the same type, partly explains, in the writer's opinion, the divergent data recorded in the literature for the composition of fresh leaves. As a result of the appreciable decrease of substances of the Dynamic Group and of the parallel losses of dry weight of the samples, the percentages of other leaf components change even if their absolute amounts actually remain unchanged during the entire period in which the weight losses occur. Since these apparent changes can lead to erroneous conclusions, several authors, especially Vickery and Pucher (310, 318), do not express their analytical determinations as percentages, but as absolute amounts contained in samples of identical fresh weights. Another method of establishing the actual chemical changes in leaf tissues is to determine carefully all the changes in dry weight, throughout the period in which the composition of the leaves is being followed by analytical determinations, and to express the amounts of substances found at each stage of the observation period in percentages of the *initial dry weight*. Thus, the actual changes during the growth of plants, during artificial culture of detached leaves, and during curing and fermentation, can be recognized.

The second reason that the study of the Dynamic Group substances is difficult, is the lack of satisfactory analytical methods which would include all of its components. The conventional methods of determining carbohydrates,\* and the procedures developed for the quantitative analysis of the organic acids in the ether-soluble acid fraction (mainly citric and malic acid)† account for 40 to 70% of the total compounds in the Dynamic Group. The remaining, analytically still unidentified nonnitrogenous substances are obviously neither carbohydrates nor do they belong to the classes of organic compounds (pectins, etc.) which are listed in the Static Group. At a later point, some conclusions will be attempted concerning the nature of these labile, unidentified components of the leaf tissue which are evidently active participants in the oxidative changes occurring throughout the entire period after harvesting.

Just as in the Nitrogen Group, extensive internal transformations take place among the components of the Dynamic Group, resulting in considerable shifts among the components.

### 3. *Metabolism of Detached, Artificially Cultured Tobacco Leaves*

Only a part of the chemical transformations in green tobacco leaves is directly related to the reactions in harvested leaves during drying and fer-

\* Unless otherwise noted, the term "carbohydrates" is used in this text for the mono- and disaccharides, the polysaccharides such as dextrin and starch, the glucose part of glucosides, and all the other compounds which, on hydrolysis with acids, yield substances analytically determinable as carbohydrates.

† Useful methods for the determination of ether-soluble acids especially in plant tissues, have been developed by Pucher, Vickery *et al.* (204, 212-215, 217, 313).



mentation. The metabolic processes involved in the growth of the plant and in the transfer of substances into and out of the living leaves are not directly related to the chemical changes during curing and fermenting, although transfer processes can considerably influence the composition of drying leaves (see page 339). This narrows our subject to metabolism in harvested mature leaves, and even of this metabolism only the "dark metabolism" need be considered. "Light metabolism," with the assimilation of carbon dioxide as its focal point, is completely suppressed in the harvested leaves, under the conditions of storage and drying to which they are subjected (see pages 324, 362 and 365).

Although there are several studies of metabolism in tobacco leaves while they are still part of the plant, only a few authors have investigated chemical changes in detached leaves, cultivated in the dark. Vickery, Pucher *et al.* (316, 318) have studied in detail the gradual transformations in detached leaves of a cigar tobacco (Connecticut shadegrown) kept in the dark, in water and in nutrient solutions, for 230 to 300 hours.

The following exterior changes occurred during this period in the dark: normal, turgid leaves (0 to about 70 hours), increasingly yellowing (about 70 to about 140 hours), developing brown spots (about 140 hours to end of period).

The following results were found:

1. No significant difference existed between chemical changes in water-cultured and in solution-cultured (glucose, ammonium salts) leaves.

2. In the *Static Group* the ash components and the cellulose and hemicelluloses (pentosans, etc.) changed, if at all, only very little in amount or nature. The ether-soluble compounds decreased by about 30%, either by transformation into ether-insoluble substances, or by loss in the form of volatile compounds.

3. In the *Nitrogen Group* the total nitrogen of the leaf tissue remained nearly constant. Small amounts (between 5 and 10% of the initial total nitrogen, or 0.2 to 0.5% of the dry weight) might have been lost from the tissues, in the form of volatile nitrogen compounds (ammonia, other volatile bases), but this hardly exceeds the limits of error involved in these experiments.

The errors in studies of this kind originate from two independent sources: (1) the analytical error, which depends on the accuracy and reproducibility of the analytical method employed for each type of determination; and (2) the sampling error, which is caused by the unavoidable necessity of selecting and analyzing, at each successive stage of the observation period, other leaves of the total sample. The latter error can be kept at its lowest possible limit by using leaves of the same origin, age, and visible characteristics, but it can never be completely eliminated, particularly not for those components of the tissue which are especially variable.

Profound changes, however, occur in the *distribution* of the total nitrogen, the main effect being a progressive digestion of the water-insoluble nitrogen compounds, the largest part of which are proteins (316, 321). This change, easily detectable as a transfer of nitrogen from the insoluble to the soluble fraction, indicates that, of the proteins initially present in the leaf, about 50% have been transformed by the end of the culture period. At this stage, the leaves contain only about 9.5% proteins, per dry weight, compared with a protein content of 15% at the start.

The final protein content of 9.5% results from the 50% loss of the original protein content of 15% and from the simultaneous loss of dry weight of the leaf (about 20%).

The nitrogen compounds in the water-soluble fraction which, as transformation products, replace the digested proteins, are partly monoamino compounds and partly ammonia compounds and amides, the last class consisting mainly of asparagine and some glutamine. How each of these groups of soluble nitrogen compounds originates from the fragments of the disappearing proteins is not yet completely clear.

The protein nitrogen, according to analyses by Vickery and Pucher (hydrolysis with 10 *N* acid, followed by analysis of the hydrolyzate), is composed of about 56% monoamino nitrogen and about 8% amide nitrogen. They did not identify the nature of the remaining 36% of protein nitrogen, but it seems not unlikely that it corresponds to the basic nitrogen (guanidine and imidazole nitrogen) of basic amino acids, such as arginine and histidine, which were contained in the complex protein molecules as structural elements (see page 340).

That tobacco probably contains considerable quantities of arginine and histidine is inferred from the composition of edestin (a plant protein), which contains similar amounts of monoamino and amide nitrogens as tobacco protein. On a percentage basis these are: monoamino nitrogen in edestin, 48 to 58%, in tobacco protein, about 56%; amide nitrogen in edestin, about 10%, in tobacco protein, about 6 to 11%. In addition, edestin contains the following nitrogens: arginine, 27%; histidine, 5.8%; lysine, 4%; cystine, 1.5%; unidentified and nonamino, about 4% (304, 316).

The amount of analytically determined amino groups of the newly formed soluble nitrogen compounds is about the same as, or somewhat less than, that of the amino groups which are set free by the breaking up of the tobacco proteins. But the ammonia and amide nitrogens, which in the cultured tobacco leaf appear in increasing amounts in the soluble nitrogen fraction, far exceed the amount of amide nitrogen which was determined analytically in the leaf protein. This indicates that the protein-digesting enzymes of the leaf are able to transform certain fragments of the protein into ammonia and into amides, although these fragments remain resistant toward hydrolysis with 10 *N* sulfuric acid, which is used as a hydrolyzing agent in the analytical determination of the amide groups of the leaf protein. It is not unlikely that these protein fragments are identical with the basic nitrogen of arginine, histidine, and related amino acids. Conceivably, the basic groups of these compounds resist acid hydrolysis but are transformed into ammonia and amides by the proteolytic leaf enzymes.

The formation of asparagine and glutamine is, according to various authors [see historical introduction in Vickery *et al.* (316)], due to reactions of intermediately formed ammonia with certain nonnitrogenous compounds (see also page 342). The formation of glutamine is small, compared with that of asparagine, in the leaves cultured in the dark.\* Vickery and Pucher favor the theory that the ammonia participating in this synthesis is partly derived from amino compounds, via oxidation and hydrolysis. Probably, the ammonia is derived from both sources (see page 342). As the culture period progresses, formation of amides diminishes, evidently due to a gradual exhaustion of their nonnitrogenous precursor substances, while ammonia (or ammonium compounds) accumulates rapidly. The relative increases in soluble amino compounds, amides, and ammonia are recorded in Table III.

TABLE III  
INCREASE OF SOLUBLE NITROGEN COMPOUNDS IN DETACHED CIGAR TOBACCO LEAVES  
CULTURED IN THE DARK (316)

Duration of culture period, hrs.	Amino acid nitrogen	Ammonia nitrogen	Amide nitrogen	
			Asparagine N	Glutamine N
	In per cent of initial dry weight			
0	0.14	0.013	0.019	0.013
100	0.58	0.042	0.158	0.056
200	0.70	0.246	0.321	0.037
Final amount exceeds initial amount by a factor of	5	18.9	16.9	2.8
Hours	In per cent of dry weights at each stage of culture period			
0	0.14	0.013	0.019	0.013
100	0.67	0.049	0.180	0.064
200	0.86	0.304	0.395	0.045
Final percentage exceeds initial percentage by a factor of	6.1	23.4	20.8	3.4

The high relative increase of ammonia, and amides, and the simultaneous digestion of the leaf proteins constitute, according to Vickery and Pucher, the main part of the chemical transformations in the Nitrogen Group during culture of tobacco leaves in the dark. Compared with these marked internal shifts, the other nitrogenous components undergo only slight alterations during the culture period. It seems that the nitrate nitrogen decreased by about 11% of its initial value (this is close to the limits of accuracy of the determinations), which, according to Vickery and Pucher, may indicate a corresponding reduction to ammonia. Nicotine also ap-

\* In cultures in the light, formation of glutamine exceeds that of asparagine (see page 361). The synthesis of both amides, from ammonia and unidentified precursor substances, is discussed in detail by Vickery and co-workers. It appears clear that only a very small fraction of the amides formed in the cultured leaves can have been derived directly from the protein as preformed fragments of the latter.

parently decreased during the culture period by about 15%. A transformation of the alkaloid into other products may have occurred, but their nature is still unknown.

The chemical changes in the *Dynamic Group* substances are even more extensive than those in the Nitrogen Group. The reactions of the nitrogen compounds in the leaves cultured by Vickery and Pucher were almost entirely restricted to internal shifts, losses of nitrogenous substances from the tissues being only minor. In the Dynamic Group, however, a considerable part of the substances disappeared by volatilization during the culture period, as shown by a considerable decrease in dry weight of the leaf samples.

Only a fraction of this weight loss can be attributed to chemical changes in the Static or Nitrogen Group. As mentioned before, after culture in the dark the ether-soluble compounds of the Static Group decreased by about 30% of their initial value (in percentage of dry weight, by about 0.3 times 6%, or 1.8%). This decrease in ether-soluble substances is probably caused by their partial transformation into ether-insoluble compounds rather than by removal in the form of volatile compounds. [Vickery and Pucher (310, 318) discuss the possibility that the loss of ether-soluble substances is partly due to a decomposition of ether-soluble chlorophyll into compounds, a fraction of which is less soluble in ether.] In order to obtain a minimum value for the loss in leaf weight attributable to compounds of the Dynamic Group, we assume here the complete volatilization of the ether-soluble compounds. Loss of pectins accounts for a further small loss of weight. This may amount to 10% of the initial value of the pectins (in percentage of dry weight, 0.9%). [This is based on data in the literature for changes of pectins during curing (see page 330). They resemble generally the changes observed by Vickery and Pucher for dark-cultured leaves, for which no pectin analyses were published.] All the other members of the Static Group remained unchanged, within the limits of analytical and sampling errors. Of the total nitrogen, about 0.5 to 0.7% of the dry weight of the leaves disappeared during the culture period, corresponding to a loss of some 3 to 4% of nitrogenous compounds in percentage of dry weight of the leaves. This decrease hardly exceeds the limits of error in the analyses. Furthermore, even if this nitrogen loss actually occurred, it would not necessarily involve the loss of the total weight of the nitrogenous compounds from which it originated. [The hydrolysis of amides and the oxidative hydrolysis of amino compounds yields ammonia which is lost at certain pH values. Such an effect results in a much smaller weight loss than that calculated on the assumption that the entire nitrogenous parent substances disappear.] Nevertheless, a 3.5% loss of nitrogen compounds will be assumed, for the purposes of this calculation.

Totaling the maximum losses of all leaf constituents other than those of the Dynamic Group, we obtain a value of about 6% of the dry weight. However, the actual dry weight losses in three separate series of leaf cultures studied by Vickery and Pucher amounted to an average of about 20%. Hence, one must conclude that during dark culture of the leaves at least 14% of the dry weight is lost through transformation of compounds of the *Dynamic Group* into volatile products.

The principal members of the Dynamic Group are carbohydrates, and ether-soluble acids (both of which can be determined analytically), and the relatively large amount of analytically undetermined substances, whose quantity is derived merely by subtracting the sum of all the known leaf constituents from the total dry weight of the samples (see Table I, page 313).

In leaves cultured in the dark, the decrease of total carbohydrates (foot-note, p. 313) was found to be 80 to 90% of the initial amount of total carbohydrates or about 3.5% of the dry weight of the leaves (Connecticut shadegrown) studied by Vickery and Pucher. The sum of the ether-soluble organic acids decreased from approximately 16 to 14% of the dry weight. Thus, the loss of analytically known compounds of the Dynamic Group totals about 5.5% of the dry weight, leaving a loss of about 8.5% unaccounted for. This loss evidently must be attributed to a transformation and final volatilization of a large part of the unidentified dynamic substances, which in fresh leaves of a cigar type tobacco amount to about 17% of the dry weight. Apparently, during the culture period, 8.5% of the dry weight, or about 50% of these substances, are transformed into volatile compounds, and an amount is left which corresponds to 8.5% of the initial dry weight of the leaves, or to about 10.5% of their final dry weight at the end of the culture period. Possibly, this remainder of unidentified substances is of a nature other than the 50% which disappears so easily during the culture period, simultaneously with the disappearance of 80 to 90% of the known carbohydrates.

The parallelism in the behavior of the carbohydrates and the unidentified dynamic substances leads one to the conclusion that both kinds of substances are closely related. This conclusion is strengthened by the finding that the metabolic reactions just discussed can be reversed. Simultaneously with the analytical studies of leaves cultured in the dark, Vickery and Pucher carried out similar experiments with detached leaves cultured in the light. These leaves, for a considerable length of time, were able to synthesize organic substances in the same way as were leaves attached to the plant; in contrast to the leaves cultured in the dark, they gained considerably in weight.

In the leaves cultured in the light, the changes of the Nitrogen Group components resembled those in the leaves cultured in the dark: proteins disappeared, and soluble nitrogen compounds were formed; glutamine appeared to a relatively higher extent, indicating that the nonnitrogenous precursor of this compound is formed in the light; nitrate decreased somewhat more, and nicotine remained almost constant. No indications of changes other than those in the dark-cultured leaves were found in the Static Group.

At the end of the culture period, the average dry weights of the leaves

cultured in the light exceeded the initial dry weights by 23%. A calculation similar to that carried out for the leaves cultured in the dark seems to show that about 21 of the 23% weight gain has to be attributed to the synthesis of substances of the Dynamic Group. Specifically, the Dynamic Group showed an increase of 12% in analytically detectable carbohydrates (formed by photochemical carbon dioxide assimilation), and of about 1% of ether-soluble organic acids. Consequently, the unidentified substances increase by an amount equal to 10% of the dry weight and the total quantity of these substances reaches 29% of the initial dry weight, or about 24% of the dry weight of the leaf samples at the end of the period of culture in the light.

Here again a close parallelism exists between the behavior of the carbohydrates and the unidentified substances of the Dynamic Group. Considering the speed and ease with which carbohydrates varied, in the above experiments, from 0.5 to 16%, and the unidentified dynamic substances from 12.5 to 24% of the dry weight, it is not astonishing that the composition of fresh tobacco leaves, even of the same plant, largely depends on the exact time of harvest, the time interval between harvest and analysis, and the conditions of storage before analysis. The high reactivity of carbohydrates in the tissues of intact plants is a well-known phenomenon. Several authors (169, 260, 302) have observed in the leaves of young tobacco plants daily periodic variations of total carbohydrates from 3 to 10% of the dry weight, and even greater changes of individual sugars and polysaccharides. (The daily variations in the leaves of older and mature tobacco plants are, as a rule, smaller, running from about 3 to 5%.) These variations, together with the basic differences between the various types of *N. tabacum*, might explain the very divergent values to be found in the literature for the amounts of carbohydrates in fresh tobacco leaves.

For the green leaves of one type of cigarette tobacco (American Krim), L'vov and Beresnegovskaja report an average content of total carbohydrates of 38% (143). Vickery *et al.* (316) found the average total carbohydrate content of green cigar tobacco leaves (Connecticut shadegrown) to amount to only 5 to 7%. One cause for these discrepancies may lie in the fact that the analytical methods for the determination of carbohydrates used by the authors are different, and may lead to results that are not identical even when used on the same sample. But the deviations resulting from this analytical factor are considerably smaller than those given above.

### III. Tobacco Curing: Its Chemical Effects

#### 1. General Characteristics

The first important step in the treatment of harvested tobacco leaves is curing. Physically, curing is a slow drying operation resulting in the loss

of large amounts of water; at the end of the curing process, the ratio of water to solids in the leaves has decreased from an initial value of about 900/100 to about 25/100, or a loss of roughly 97% of the water content of the green leaves (82, 109, 332). Along with the slow drying, considerable chemical changes take place. These are indicated by various external signs: the leaves develop a distinct odor; they lose their glossy ("gummy") appearance; their color changes from green, to light green, and then to a bright yellow which, in the air-cured leaves (but not in the flue-cured), gives way to progressively darker brown coloration. Chemical analysis proves that significant transformations accompany these visible changes. As a result of curing, the dry weight decreases by about 15 to 25% (56, 263), a very considerable loss of solid substances from the leaf tissues. In the first stage of curing the leaves are still alive; they are in a state of gradual starvation similar to that of the leaves in the dark-culture experiments of Vickery and Pucher. After the loss of water has reached a certain point, all life processes in the leaves stop, and the destruction of chlorophyll and other characteristic changes predominate.

The method of curing is decisive for the kind and extent of the chemical changes during the drying operation (78, 80, 82, 342). The three principal methods used are air curing, flue curing, and fire curing. In some cases sun curing and presweating are preliminary steps to any of these methods.

There are also types of curing employed which lie between those listed above, and some rarely used, divergent methods [e. g., perique tobacco is air cured for a short time, and then fermented in its own juice (77)], but the greatest part of smoking tobacco is cured by one of the three principal methods mentioned above.

The main differences of the three curing methods are the speed with which drying is accomplished, and the temperatures used for this purpose. The different speed and intensity of drying determine the point at which the life processes and enzymic reactions in the leaf tissues are interrupted. Accordingly, different chemical pictures result from the different methods of curing.

In the following description of the chemical changes occurring with air curing, additional data are included concerning the chemical nature of the chemical components of the leaf tissues.

## 2. *Air Curing*

The slowest and mildest drying procedure is air curing.

The tobacco is suspended so that it hangs free, in sheds which permit a regulated flow of outside air, and the drying results from natural loss of water which occurs during the wilting and further desiccation of the leaves. The time required for completion of this

process (until the leaves contain only about 20% water per dry weight) depends greatly on weather conditions, such as temperature, relative humidity, and movement of air. In air curing, a very gradual drying of the leaves is desirable (except tobacco crops grown in a very wet season, which show a high tendency to rot), with a correspondingly prolonged period during which the leaf tissues remain sufficiently moist to permit chemical and enzymic reactions to proceed freely. In air curing, the tobacco, as a rule, reaches its yellow stage about 12 days after the harvest, its brown stage after about 6 more days, and the fully cured stage after an additional 35 days. These time intervals vary, of course, with the weather and type and crop of tobacco.

There is a growing tendency to standardize air curing by artificial means, as, for example, by heating the curing sheds on very humid days to remove the excessive moisture from the air, or by air conditioning, throughout the curing period, sheds in which particularly valuable crops, such as wrapper cigar tobaccos, are being cured (35, 79).

Some tobaccos are harvested as whole plants and are cured with the leaves still attached to the main stalk, while in other types the leaves are primed and hung in a vertical position on strings for curing. These two kinds of air curing lead to a difference in chemical composition by the end of curing. Some tobacco types are exposed, before curing in the shed, to the sun and open air (sun curing); in others, the freshly harvested

TABLE IV  
CHEMICAL CHANGES IN PRIMED, AIR-CURED CIGAR TOBACCO LEAVES\*

Constituent	Amt. in per cent of initial dry wt.		Changes, in per cent of		Amt. in per cent of dry wt. of cured leaves
	Before curing	After curing	Harvest dry wt.	Initial amt. of constituent	
Ash (inorg. cations and anions)	14.0	14.0	0	0	16.5
Crude fiber (cellulose and lignin)	9.5	9.5	0	0	11.3
Pentosans	3.0	3.0	0	0	3.5
Pectins	7.0	7.0	0	0	8.5
Ether-sol. compds. (volatile oils, resins, waxes, paraffins)	7.0	6.0	-1	-14	7.0
Tannins (polyphenols, phenolic acids)	2.5	2.5	-0.7†	-28†	3.0
Oxalic acid	2.0	2.0	0	0	2.5
Total nitrogen	4.1				4.7
Nitrogenous compds. (proteins, amino acids, amides, ammonia, nitrates, alkaloids)	24.0	23.0	-1	-4	27.0
Carbohydrates (poly- and monosaccharides, starch, dextrin)	3.0	1.0	-2	-67	1.2
Ether-sol. org. acids (citric, malic, unidentified)	11.0	10.0	-1	-9	11.5
Unidentified dynamic compds.	17.0	7.0	-10	-53	8.2
Total	100.0	85.0	-15		100.0

\* Air-cured cigarette tobacco leaves show similar conversions and losses.

† These decreases are merely transformations into other constituents, and are not losses of substance by the leaves.



leaves are packed in piles for a short period (presweating), as a preliminary to shed curing. Both these methods are used for some domestic (Virginia sun-cured) and oriental cigarette tobaccos, but they are gradually being replaced by methods permitting a better control of the drying rate.

With very few exceptions, the cigar tobaccos are air cured, while a large part of the cigarette tobaccos is flue cured. This difference in treatment enhances the specific differences inherent in the two types of tobacco, and when combined with the subsequent specific treatments, results in the chemically different alkaline type tobacco (cigar) and the acid type tobacco (cigarette). There are a number of cigarette tobaccos (for example, mak-horka, Maryland, and Burley), however, which are treated by a more or less modified air-curing process (77).

Tables IV and V give the average chemical composition of a cigar type tobacco before and after air curing. The values listed are no more than rough averages for one typical tobacco type. The principal chemical reactions which result in the changes listed in the two tables are mainly hydrolyses and oxidations transforming high molecular proteins into simpler

TABLE V  
CHEMICAL CHANGES IN CIGAR TOBACCO LEAVES AIR CURED ON THE STALK

Constituent	Amt. in per cent of initial dry wt.		Changes, in per cent of		Amt., in per cent of dry wt. of cured leaves
	Before curing	After curing	Harvest dry wt.	Initial amt. of constituent	
Ash (inorg. cations and anions)	14.0	14.0	0	0	18.7
Crude fiber (cellulose and lignin)	9.5	9.5	0	0	12.7
Pentosans	3.0	3.0	0	0	4.0
Pectins	7.0	6.0	-1	-14	8.0
Ether-sol. compds. (volatile oils, resins, waxes, paraffins)	7.0	5.0	2	-28	6.7
Tannins (polyphenols, phenolic acids)	2.5	2.5	-0.7*	-28*	3.1
Oxalic acid	2.0	2.0	0	0	2.7
Total nitrogen	4.1				3.7
Nitrogenous compds. (proteins, amino acids, amides, ammonia, nitrates, alkaloids)	24.0	17.0	-7	-25	22.7
Carbohydrates (poly- and monosaccharides, starch, dextrin)	3.0	0.5	-2.5	-80	0.7
Ether-sol. org. acids (citric, malic, unidentified)	11.0	10.0	-1	-9	13.4
Unidentified dynamic compds.	17.0	5.5	-11.5	-63	7.3
<i>Total</i>	100.0	75.0	-25		100.0

\* These decreases are not losses of substance by the leaves.

nitrogenous compounds, and degrading carbohydrates and related compounds to volatile oxidation products. As mentioned above, there are considerable differences in the final composition of tobacco leaves, depending on their curing as single leaves or on the stalk. Nevertheless, the actual chemical changes during both types of curing differ only little, if at all. The stalk-cured leaves show a considerably higher loss of dry weight than the primed cured leaves.

This is caused mainly by a *migration* of soluble compounds, particularly of inorganic and of soluble nitrogenous substances, from the leaf blades via the midrib into the stalk. This migration, which is the result of a gradient of osmotic pressures between different parts of the drying plant, has been confirmed by analyses which determined quantitatively the increase of substances in the stalks of curing tobacco plants (108). Johnson and Ogden (109) found that the stalks of tobacco plants, analyzed immediately after harvest, removal of the leaves, and a rapid drying process, contained about 5% less ash than after the stalks had been left to dry slowly with the leaves attached. Some of the ash constituents increased even more, *e. g.*,  $K_2O$  (by 8%),  $P_2O_5$  (by 35%),  $SO_3$  (by 16%), whereas others ( $MgO$  and  $CaO$ ) remained constant. This seems to indicate a preferential migration of constituents. The total nitrogen in stalks dried with leaves attached was about 30% higher than in stalks which were rapidly dried and analyzed immediately after the harvest. These observations agree with the finding (82) that unattached leaves lost, on an average, 13.4% of the dry weight during curing, compared with a 26.6% loss in leaves of the same batch of tobacco cured on the stalk.

The chemical and enzymic processes in the leaf tissues from the start of air curing to the point when the leaves turn yellow correspond closely to the reactions observed in dark-cultured leaves (see page 317). These processes, with some additional oxidative reactions, continue during the next phase and until toward the end of curing.

**Effect on the Static Group.**—(a) *High-Molecular Carbohydrates and Pectins.*—No signs of significant changes in the cellulose or lignin compounds have been found (as to certain minor changes, see page 329). Values for crude fiber found before and after curing (106, 208, 310) are practically identical, with percentage values increasing as total weight decreases in the course of curing.

The *pentosans* which, in the form of polymerized pentoses, constitute a part of the cell walls, are comparatively inert, and show practically no change (5, 32, 54, 74, 76, 120, 125, 131, 270). More reactive than these highly polymerized carbohydrates are the pectins, a part of which are in an easily soluble form in the tobacco leaf tissues (7, 81, 112, 123, 172).

According to investigations of Ehrlich (59), the main constituent of pectins is a polyuronic acid formed by the linkage of four molecules of galacturonic acid in a ring-shaped molecule in which the aldehyde group of every single molecule of galacturonic acid is condensed with one of the hydroxyl groups of another galacturonic acid molecule (tetra-

galacturonic acid). The four carboxyl groups of this acid are partly present in the form of methyl esters (two methyl alcohols per one tetragalacturonic acid) (63, 186), and partly combined with *l*-arabinose and *d*-galactose (one each per one tetragalacturonic acid) and with acetic acid (two per one tetragalacturonic acid). This pectic acid, combined in the plant tissues with the pentosan araban, easily loses the acetic acid and carbohydrate components, thus setting free the carboxyl groups with which these fragments were linked.\* The carboxyl groups can then combine with two further molecules of methyl alcohol, by an ester linkage. By the specific action of pectase (114), some of the methoxy groups can be split off in the form of methyl alcohol. The typical properties of all these mutually related pectin substances—*e. g.*, solubility in hot and cold water, tendency to form gels, viscosity of the solutions—depend largely on slight differences in their chemical composition, particularly on the presence of carboxyl groups of tetragalacturonic acid, either as such, or in the form of methyl alcohol esters. The more methyl alcohol there is in a pectic compound, the higher its tendency to form a gel. Pectin gels of a different nature are formed by the metal salts of the tetragalacturonic acid, especially by the calcium and magnesium salts. It is understandable, then, that this changeability complicates the analytical determination of the pectic substances, and that the amount of substances determined as pectins depends on the analytical method employed. An important analytical factor is the method of extraction, since the pectins are present in the leaves in many forms, ranging from easily soluble substances to highly polymerized compounds associated with the cellulose fibers in the leaf framework.

The average pectin content, determined by precipitation with alcohol of a leaf extract prepared with ammonium oxalate solution, saponification of the precipitated crude pectins, and transformation into the calcium salt of tetragalacturonic acid, has been found, on the average, to equal about 9% (7, 32, 81, 111, 113, 199, 245) of the dry weight. Other analytical methods, based on decomposition of the pectins and determination of the fragments (methyl alcohol, furfural, carbon dioxide), give higher values, on the average, sometimes as high as 18 to 21% of the dry weight (75, 107, 229). It seems possible that the latter methods include, in addition to the pectins proper, some related leaf substances. Such an explanation of the dissimilar pectin values of different tobacco types must remain inconclusive as long as no thorough comparative pectin determinations by the various analytical methods have been made on samples of the same tobacco.

Since the majority of the high pectin values reported have been for oriental cigarette tobaccos, it is also conceivable that these types actually contain larger amounts of pectins than other tobaccos. The same oriental cigarette tobaccos are known to contain less lignin, which might explain their higher pectin content provided one concedes the possibility of a mutual substitution of the two leaf constituents. The lower average values for the pectin content of tobacco leaves have been used for the tables in this review, because most of the values recorded in the literature are centered around these lower val-

\* A critical discussion of the concepts of Ehrlich on the chemical structure of pectin is given by H. Bock in *Die Methoden der Fermentforschung*, E. Bamann and K. Myrbäck, ed., Vol. 1, Thieme, Leipzig, 1941, p. 242.

ues. Furthermore, the analytical method used to obtain the lower values seems to yield more closely reproducible results than the methods by which the higher values were obtained. Many pectin analyses (unpublished) carried out in the writer's laboratory for Pennsylvania seedleaf cigar tobacco by the method leading over the calcium salt gave the average pectin content as 8.5%.

There is no doubt that moderate changes take place in the pectins during air curing. Neuberg and co-workers found (121, 175, 181, 182, 199) that, under the influence of specific enzymes, the pectins split off part of the esterified methyl alcohol during the gradual drying of tobacco leaves, the process, however, depending on the speed with which the curing tobacco lost its moisture. The faster the drying of the leaf tissues, the smaller were the amounts of liberated methyl alcohol. This effect is obviously caused by a shortening of the period during which the water content is high enough for the pectase to exert its enzymic action. Various other reactions are similarly sensitive to the water content, for example, the transformation of chlorophyll, the oxidation of phenolic compounds, and the oxidative transformation of sugars and related compounds. A thorough study of the chemical changes in curing tobacco leaves must therefore be carried out under carefully controlled moisture conditions in order to yield reproducible and comparable results. The largest decreases of methyl alcohol observed during the drying of leaves under curing conditions were from 0.6 to 0.49% of the initial dry weight (that is, from 60 to 80% of the initial pectin methyl alcohol). In other samples, however, which may have been dried with greater speed the pectin-bound methyl alcohol remained practically unchanged. The liberated methyl alcohol is lost (under the conditions of air curing) from the leaves by evaporation. Part of the methyl alcohol seems, however, also to enter into reactions in the leaf tissues. Andreadis (1) found that, simultaneously with pectin methyl alcohol losses, there occurred considerable gains in *lignin methyl alcohol*, indicating that under certain conditions lignin-like substances can be formed from pectins (209), or at least with participation of pectin fragments. It is conceivable that the loss of elasticity and increasing brittleness of overcured tobacco leaves are the outward signs of such chemical changes.

As for pectin compounds other than pectin methyl alcohol, Andreadis (1) found that, as a result of curing, the carbon dioxide which can be split off the galacturonic acid decreased by about 10% of its initial value. This leads one to conclude that, during drying, part of the tetragalacturonic acid is hydrolyzed into the monomer acid, which is decarboxylated. It is possible that some of the tobacco leaf pentoses and pentosans are produced by processes of this kind from substances belonging to the pectin group (32).

The over-all picture shown by the pectins seems to indicate that these substances are relatively more reactive than the other members of the Static Group, and that under the influence of specific leaf enzymes and favorable moisture conditions during curing, up to 10% of the total pectins (or about 0.9% of the dry weight of the green tobacco) undergoes a more or less thorough decomposition.

(b) *Ether-Soluble Substances*.—Another class within the Static Group is that of the ether-soluble substances. This group consists of a number of compounds—some still unidentified—of a very heterogeneous chemical nature, their only common property being a readier solubility in diethyl ether and other organic solvents than in water. Thus, as in many other instances of the analysis of natural products, ether extraction of tobacco leaves is more a conventional and comparative method than a determination of well-defined chemical compounds. Among the ether-extractable substances are not included those components of tobacco which, in a strongly acid medium, form free acids that are ether-soluble (*e. g.*, citric or malic acid), or those which in a strongly alkaline medium form ether-soluble bases (*e. g.*, tobacco alkaloids). At the normal pH (4 to 7) of tobacco leaf tissue, these compounds are present almost completely in the form of salts which are easily soluble in water and not directly extracted by ether.

Depending upon the pH of the leaf tissues, and as a result of the hydrolytic dissociation of some of these salts, *e. g.*, for nicotine [see Vickery and Pucher (307)], very small amounts of the hydroxy acids and tobacco alkaloids are present in a free form in the leaves, and are extracted by ether, together with the typical ether-extractable substances. The largest part of these acids and alkaloids, however, remains in the tissues and is extracted only after admixture of strong acid or strong alkali.

The main components of the ether extract are hydrocarbons (paraffins and some terpenes), alcohols (aliphatic, terpene and resin), acids (saturated and unsaturated fatty acids, phenolic and resin acids), esters composed of combinations of the alcohols and acids mentioned (monohydric alcohol esters of aliphatic acids, *i. e.*, waxes), glycerin esters of saturated and unsaturated fatty acids (fats and oils), and resin esters which are composed of resin alcohols and simpler acids, or of simpler alcohols and resin acids, sterols, and smaller amounts of aldehydes. Furthermore, there are considerable amounts of high molecular polymerization products of an unidentified nature among the ether-extractable substances. In view of the complexity of this mixture—varying in nature with each tobacco type and with each crop—the very incomplete knowledge of these leaf components is hardly surprising, and a detailed comparison of the various analytical results obtained for this fraction of the leaf constituents would be misleading.

Even more than in the case of the pectins, both the amounts extracted from the leaves and the composition of the extracts depend on the solvent. Hydrocarbon solvents, like petroleum ether and benzene preferentially extract the paraffins, waxes, and certain resins. Alcohols or esters, however, extract these compounds to a smaller extent but are good solvents for many resin alcohols, resin acids, resin esters, and, in general, other substances containing a relatively large percentage of oxygen in their molecules (215, 219). The properties of diethyl ether as a solvent lie between these two extremes. The separation of the various types of compounds contained in the mixture (the ether extract) is based on three factors: (1) difference of behavior toward solvents; (2) selective extraction of acidic substances from the organic solvents with aqueous solutions of increasing alkalinity; and (3) distillation methods.

The *volatile oils* fraction can be isolated by steam distillation. These oils are secreted mainly by the gland hairs of the tobacco leaves and are contained in the sticky "gum" which covers the surface of the leaves. They have been studied by many authors (29, 32, 50, 92, 93, 102, 122, 135, 197, 201, 234, 235) because of their importance as contributors to the specific aroma of smoking tobacco. The total amounts of volatile oils are relatively small, varying between about 0.07 and 0.5% of the dry weight of the leaves.

As a rule, the lower, *i. e.*, the older, leaves contain considerably less oil than the upper, or younger, leaves. The values found (234) in one tobacco type are 0.16 and 0.47%, respectively, for the two kinds of leaves. A dry season produces more volatile oils than a wet season. Among the organic acids contained in these volatile oils, in small amounts in a free state, the remainder in an esterified state, the following have been identified: formic, acetic, isovaleric, isobutyric, caprylic, palmitic, myristic, and terephthalic acids. Among the alcohols found partly free, partly esterified with these acids, are:  $C_4$  and  $C_6$  aliphatic alcohols, terpene alcohols, and polyhydric alcohols such as glycerin and sorbitol (139, 180, 234, 237). Furfuryl alcohol and furfuraldehyde were also found. Of the phenols, eugenol and isoeugenol were identified (201) as components of the volatile oils. The steam-distillable hydrocarbons, together with the remainder of the volatile oils, contain (93) the compounds  $C_{10}H_{18}$ ,  $C_{10}H_{16}$ , and  $C_{11}H_{20}$  (92), probably representatives of the class of terpenes (limonene) and the paraffins  $C_{27}H_{56}$  and  $C_{31}H_{64}$  (102). The characteristic constants of the volatile oils (acid numbers, ester numbers, iodine numbers, etc.) indicate: (1) that the main part of the oils consists of esters; and (2) that many of the compounds contain unsaturated bonds (234, 235).

Evidently, once the volatile oils have been secreted by the leaf, they no longer participate in its metabolism. According to Kurilo (135), the amount of volatile oils increases during curing. It seems possible that this is due to the gradual formation of volatile esters from the less volatile acids and alcohols (resin part) of the ether-soluble fraction. The changes of the characteristic number of the volatile oils indicate that the curing causes further esterifications and saturation of double bonds in the oils (135, 233). When distilled from the leaves or leaf extracts, the volatile oils

change rapidly; they lose their specific, agreeable aroma, and are transformed into acidic compounds, with the simultaneous formation of resins. Exclusion of oxygen prevents these changes which, obviously, are caused by autoxidations and polymerizations of the reactive components of the volatile oil fraction. In the tobacco leaves, the volatile oils seem to be protected against this rapid autoxidation, even during the drying process. Possibly, this is due to the reducing properties of accessory leaf components. Thus, the volatile oils withstand the curing phase without any considerable losses, although finer internal changes seem to take place which, as a rule, lead to a favorable change in the specific aroma of the leaves (197, 202, 235).

The main part of the ether-extractable substances—the less volatile compounds—is usually referred to as the *tobacco resins*. Most of them are chemically related to the volatile oils. The lower volatility of the resins is due (1) to a smaller degree of esterification as compared with that of the oils, and (2) to the presence of higher molecular substances, which are partly oxidation and polymerization products of members of the oil group, partly individual compounds of a low volatility not contained in the oils. The resins vary in amount between 5 and 10% of the dry weight (50, 81, 119, 133, 201, 249, 314), and average about 7% for cigar tobacco, and about 7.5% for cigarette tobacco.

Dry growing seasons increase the resin content (40). Of the total resins, about 6% (0.43% of the dry weight) are waxes (133, 237), and about an equal amount, paraffins. Some authors found liquid oils in the resin fraction, in addition to the waxes. Oleic, linoleic, linolenic, palmitic, and myristic acids were identified, mostly in the form of esters of mono- and polyhydric alcohols (102, 133, 232, 237). Among the paraffins, hydrocarbons with 27 and 31 carbon atoms were isolated. The resins are largely composed of the same types of terpenes, alcohols, and acids as those listed above as components of the volatile oils (203). Some hydroxy and phenolic acids (quinic, caffeic, and chlorogenic acids) (123, 124), contained in the resinous part of the ether extracts, are not found in the volatile oil fraction.

No sharp border line exists between the volatile oils and resins. Both of them form mutual solutions, whose separation largely depends on the method of distillation used to remove the volatile oils. Little is known of the polymerized components of the resin fraction, except that some higher molecular acids ( $\alpha$ - and  $\beta$ -tabacen acid, empirical formula about  $C_{24}H_{41}O_6$ ) are present (50, 235), and that a further portion, known as resenes and including the  $\gamma$ -tabacen acid, is insoluble in diethyl ether and soluble only in benzene and petroleum ether, an indication that these polymerization products are relatively poor in chemically bound oxygen. Symons (299) re-

ports, for flue-cured cigarette tobaccos, contents of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -acids as high as 2.1 to 5.9% of the dry weight.

The resins can be regarded as both a source and a transformation product of the volatile oils. Esterification of some of the alcohols with some of the acids of the resin fraction can yield volatile compounds, and a mild heating of the resins results in a partial decomposition, with the formation of volatile oils. On the other hand, a part of the volatile oils is polymerized, under the influence of oxygen, to resinous products. Thus, the presence of a constant proportion of volatile oils in tobacco leaves does not necessarily prove a high stability of the oils in this environment, but might also be explained as due to a state of balance between their formation from certain resinous substances, and their transformation, by way of polymerization, to resins of another type. Despite many efforts to develop a satisfactory, detailed analysis of tobacco resins, our present knowledge of the composition and reactivities of these substances is still far from complete. The total ether-soluble fraction decreases, as a rule, during the drying of tobacco leaves (310). This decrease, on an average, is about 20% of the initial amount of the resins, or about 1.4% of the dry weight.

At the same time, determinations of the characteristic numbers of the resins reveal that the amount of esters increases as a result of the air curing, while the number of unsaturated bonds decreases (69, 249).

How much the decrease of ether-soluble compounds is due to an actual loss of dry substance from the leaves, either by volatilization or mechanical losses,\* and how much to a transformation into compounds of a diminished solubility in ether, has not yet been answered clearly.

(c) *Plant Pigments*.—A further class of substances, small in absolute amount, but important for metabolism in the light and for the appearance of the tobacco leaf, is that of *plant pigments*. The complex nature of most of these pigments makes it difficult to place them in any one of the groups into which the leaf components have been subdivided in this review. *Chlorophyll*, for example, in its esterified form, belongs with the components of the ether extract, although it is easily converted by the splitting of its phytol ester into compounds of a greatly diminished solubility in ether. The nitrogen content of its pyrrole rings brings chlorophyll into the Nitrogen Group. Its nitrogen content, however, is low, about 6.2%, or only 0.019 to 0.25% of the dry weight of the green leaves. Of the yellow pigments which have been found in tobacco leaves, *carotene* belongs to the

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\* Some components of the volatile oils and resins form a gummy coating on the leaf surface. This coating is loosened and becomes brittle, as a result of effective air curing. Possibly, a certain percentage of the resinous materials is thereby lost.



unsaturated hydrocarbons, and, therefore, must be listed among the ether-extractable substances; *xanthophyll*, which contains oxygen, is a member of the same class. The flavone pigments, which, almost without exception, contain two phenolic groups in the *o*-position, belong with the phenols and polyphenols.

The amount of chlorophyll in tobacco leaves roughly parallels the total nitrogen content, and particularly the protein content (86, 145, 151). The amount of chlorophyll (expressed in per cent of dry weight) varies between 0.3 and 4.0% (57). The total amount of the yellow pigments (carotene and xanthophyll) in mature leaves is about one-fifth to one-third that of chlorophyll (170). If tobacco leaves are dried very fast, they retain practically all their chlorophyll. This is another example of the "freezing" of chemical and enzymic changes by rapid desiccation of the tissues (336). However, in the normal course of air curing, the chlorophyll content steadily decreases (11, 13). Russian investigators (198, 231, 355) observed the following changes for air-cured cigarette leaves:

Phase of curing	Plant pigments, color	In per cent of their initial amounts		
		Chlorophyll	Xanthophyll	Carotene
Initial state	Green	100	100	100
After 3 days' curing	Light green	61	78	99
After 5 days' curing	Yellow	17	57	94
After 12 days' curing	Brown	6	46	49

The initial stage of chlorophyll decomposition is the splitting of its ester linkages with the formation of phytol and methyl alcohol. The further destruction of chlorophyll is due to oxidation, which can be avoided by the exclusion of air (337). The final oxidation products of the chlorophyll in tobacco leaves have not yet been investigated in detail. However, it is likely that these products consist of derivatives of the imide of maleic acid (64, 65, 227), with porphyrin compounds as intermediates of the destruction process. Only a very small amount of chlorophyll remains in the leaf tissues by the end of air curing, which explains the disappearance of the green color of the leaves.

Carotene and xanthophyll, considerably more stable under air-curing conditions than is chlorophyll, cause the yellow color which appears in the middle of this process. At the end of air curing, these pigments, too, decrease, probably because of enzymic oxidations.

(d) *Phenols, Polyphenols, and Tannins*.—The brown color which finally replaces the yellow in air-cured tobacco leaves is caused by the transformation of substances of the phenol, polyphenol, and tannin classes.

A number of the tobacco leaf components contain phenol groups, possibly originating from the sugars, via the formation of cyclic systems (hydroaromatic hydroxy compounds), and dehydrogenation of the latter. In fact, the cyclic sugar, inositol, has been identified in tobacco (up to 1.36% of dry weight) (26, 246, 261), as well as quinic acid (124, 246), a carboxylated tetrahydroxy acid of the hydroaromatic series. The total amounts of phenolic compounds in tobacco leaves are still uncertain. Some authors have accepted determination of the reducing substances present in leaf extracts, aside from the carbohydrates, as a method yielding approximate values for total phenols and tannins.

This method can give only the upper limits for the concentration of phenolic compounds in the tissues, because of the presence, in the extracts, of nonphenolic, reducing substances, in addition to the carbohydrates and the phenols, *e. g.*, ascorbic acid, which can amount to 0.5 to 1% of the dry weight (85). A second method is based on permanganate titrations of the reducing substances in tobacco extracts, before and after the removal of polyphenols and tannins by adsorption on hide powder. This method, too yields only approximate values for the total amounts of phenolic substances (32).

Both of these summary methods give amounts of the phenolic compounds contained in the leaf tissue ranging between 1.8 and 5.2% of the dry weight (7, 200, 201, 240, 241, 250, 305) with the average at about 2.7%. The following representatives of the phenolic components have been identified as constituents of tobacco leaf:

1. Simple phenols, distillable from acidified extracts (171). Amounts, after curing, were found to range between 0.01 and 0.04% of the dry weight. These volatile phenols may include catechol, eugenol, and isoeugenol, as well as related compounds (171).

2. Glucosides of phenolic compounds, particularly rutin, the rhamnogluco-  
side of quercetin 1,3,3',4'-tetraoxyflavonol (16, 38, 95, 134, 177, 178, 179). Compounds of this type (flavones, flavonols, anthocyanins, and their glucosides) are commonly listed among the plant pigments. The amount of rutin in the green tobacco leaf varies between 0.25 and 1.7% (134). In flue-cured tobacco it varies between 0.008 and 0.61%, with 0.4% as an average for good quality leaves (37, 38).

3. Phenolic acids, such as caffeic and chlorogenic acids, the latter a depside of caffeic and quinic acid. Their amounts in the tobacco leaf vary considerably: German tobacco contains up to 2.4 to 4.5% (124), while other tobaccos seem to contain much smaller amounts.

In view of the sensitivity of phenolic compounds to oxidation, comparative values for different tobaccos can be determined only for leaves which have the same history of treatment.

#### 4. High molecular substances of a phenolic nature (the tannins).

Since the transformation of most of the other phenolic compounds into oxidation products is a result of air curing, the small amounts of volatile phenols found in cured tobacco samples probably represent only a fraction of the amounts present in the green leaf. The former effect has been proved for the phenol glucoside, rutin. Neuberg and Kobel (177-179) found as much as 1.7% rutin in green tobacco leaves, but after air curing the leaves contain only traces, or none, of this substance (37). The product of the enzymic oxidation of rutin is dark brown in color (179). According to various authors (179, 339), this oxidation product of rutin is what causes the tobacco to darken in the final stages of air curing, possibly together with the oxidation products of other phenolic compounds, such as caffeic and chlorogenic acids.

The polyphenols and tannins are the polymerization and oxidation products of simpler phenolic compounds. They still contain some phenol groups. As a rule, the tannins also contain carbohydrates (bound by glucoside linkages), and nitrogenous compounds (combined with the tannins by the same groups which cause their tanning action on proteins). Complete disappearance of the phenolic groups by exhaustive oxidation yields, as reaction products of the tannins, high molecular brownish substances which resemble, in many of their properties, the humins and humic acids.

The fact that the phenolic compounds are relatively stable in the tissues of the green tobacco leaf is explained by some authors (339) to be the protective action of accessory, strongly reducing components of the leaf tissues (*e. g.*, ascorbic acid). Roberts (224, 225), however, assumes that the stability of the phenolic compounds in the green leaf is due to the fine structure of the latter. According to this theory, the enzyme causing the oxidation of phenols and polyphenols (polyphenol oxidase or cytochrome oxidase) (225), is contained in the cytoplasm in the cells of the leaf, whereas the phenolic compounds are in the extracellular medium.

Roberts has developed this theory in analogy to his thorough studies of tea fermentation, a process in which the oxidation of polyphenols and tannins (22.2% of the dry weight) is much more pronounced than in tobacco leaves (224).

As long as the cell membranes are intact, the phenols and oxidases remain separated and no oxidation can occur. But when the chemical changes resulting from air curing have caused a corrosion of the cell membranes, contact between the phenols and the oxidase is established, and oxidation takes place.

Such a corrosion of the cell walls is likely to occur as a result of the partial decomposi-

tion of pectins, pentosans, resinous substances, and other materials which, besides cellulose and lignin, are components of the cell membranes.

(e) *Oxalic Acid*.—Although it belongs, chemically speaking, with the organic acids included in the Dynamic Group, oxalic acid is listed in this survey with the substances of the Static Group, because of the slight changes it undergoes during all stages of tobacco treatment, including curing (90). The oxalic acid content of normal leaves varies between 1.5 and 3.7% of the dry weight, with the average at about 2.5% (81, 119, 201, 231, 238, 243, 311, 313, 314, 316). Some cigarette tobaccos contain considerably less. The stability of oxalic acid in leaf tissues, in spite of its known susceptibility to oxidation to carbon dioxide and water, may be due to the fact that it exists in leaf tissues in the form of the insoluble calcium salt (313). It is possible that any oxalic acid formed in excess of this calcium-bound amount is oxidized so rapidly that it never accumulates in detectable quantities.

**Effect on the Nitrogen Group.**—(a) *Its Conversions*.—The nitrogenous substances undergo chemical changes during air curing which are very similar to those observed in tobacco leaves cultured in the dark (see pages 318–320).

A summary of the changes of total nitrogen, as a result of air curing, is listed in Tables IV and V. Only small losses of total nitrogen are observed in primed cured tobacco leaves. These losses range from quantities too small to be detected up to about 0.3% nitrogen per dry weight, or about 7.5% of the total nitrogen contained in the freshly harvested, uncured leaves (82). Possible reasons for these losses will be discussed later. In contrast to this moderate decrease of total nitrogen in detached leaves, the losses in tobacco leaves cured on the stalk are larger. Considerable amounts of substances migrate from the leaf blade back into the stalk during the curing process (see page 327), and soluble nitrogenous substances represent a considerable portion of these migrating leaf constituents. The losses of total nitrogen in leaves cured on the stalk range between 0.6 and 1.2% of the dry weight, or 15 to 30% of the total nitrogen of uncured tobacco leaves (82, 90). Migration of soluble nitrogen compounds occurs in the intact tobacco plant, and, coupled with the decomposition and resynthesis of proteins, it provides a transportation system for moving and fixing nitrogen compounds at all points of the organism at which they are required (193). Whereas in young and growing leaves the amounts of total nitrogen are kept fairly constant by a dynamic equilibrium between influx and withdrawal of soluble nitrogenous compounds, and between digestion and resynthesis of proteins, withdrawal and digestion predominate in

senescent leaves. This effect partly explains the low values of total nitrogen and proteins in those cigarette tobaccos primed as overripe leaves (for other possible explanations, see page 314). Not all the cigarette tobaccos have a low nitrogen content; the total nitrogen and protein values recorded for makhorka are, on an average, even higher than those of typical cigar tobaccos. This is probably due to specific properties of this strain and special conditions of fertilization and harvesting (84, 323).

Signs of a migration, on a small scale, of soluble nitrogen compounds during drying can be detected even in the primed leaves, some nitrogen compounds being shifted from the leaf blade into the midrib of the leaf (82). But compared with the extensive migration into the stalk, it is of minor size, the capacity of the midrib to take up soluble substances being small compared with that of the stalks. This shift might, therefore, be called a stopped nitrogen migration.

The main factor in the mutual conversions of nitrogenous compounds is the enzymic digestion of part of the leaf proteins, with the formation of soluble nitrogen compounds. The major groups of the water-soluble nitrogen fraction as found before and after the curing (19, 25, 32, 82, 310, 318) are recorded in Tables VI and VII. Like the chemical changes observed for tobacco leaves cultured in the dark, a sharp increase in ammonia, amides, amino acids, and "rest" (mainly heterocyclic and humin) nitrogen

TABLE VI

TYPICAL CHANGES DURING CURING OF THE NITROGENOUS COMPOUNDS IN PRIMED CIGAR TOBACCO LEAVES\*

Type of nitrogen	Contents, in per cent of					Changes, in per cent of	
	Dry wt. of harvested leaves		Dry wt. of cured leaves	Total nitrogen		Dry wt. of harvested leaves	Initial amt. of compd.
	Before curing	After curing		Before curing	After curing		
Total	5.61	5.34	6.51	100.0	100.0	-0.27	-4.8
Protein (insol.)	3.69	1.65	2.01	65.8	30.9	-2.04	-44.7
Soluble	1.92	3.69	4.50	34.2	69.1	+1.77	+92.2
Amino	0.23	0.80	0.98	4.1	15	+0.57	+348
Ammonia plus amide	0.15	1.07 (1.34)†	1.30	2.7	20	+0.92 (+1.19)†	+613 (+793)†
Alkaloid	0.35	0.32	0.39	6.2	6	-0.03	-8.4
Nitrate	0.63	0.77	0.94	11.2	14.5	+0.14	+22
"Rest"	0.56	0.73	0.89	10	13.6	+0.17	+31

\* Data derived from two extensive series of experiments by Vickery and Pucher (310, 318) on the chemical changes occurring in the curing of Connecticut shade-grown cigar tobacco.

† These figures represent the sum of ammonia and amide nitrogen found in the soluble part of the cured leaves, plus the lost "total nitrogen." The assumption made in this evaluation is that the disappearing nitrogen consists exclusively of ammonia.

results from disintegration of the protein molecules. Comparison of the data in Tables VI and VII suggests that the migrating nitrogen consists primarily of amino acids, which move, in the course of curing, from the leaves into the stalk, and of relatively smaller fractions of ammonia compounds, amides, and nitrogen compounds not yet identified analytically, which are also soluble products of the protein digestion.

TABLE VII

TYPICAL CHANGES DURING CURING OF THE NITROGENOUS COMPOUNDS IN CIGAR TOBACCO LEAVES CURED ON THE STALK\*

Type of nitrogen	Contents, in per cent of					Changes, in per cent of	
	Dry wt. of harvested leaves		Dry wt. of cured leaves	Total nitrogen		Dry wt. of harvested leaves	Initial amt. of compd.
	Before curing	After curing		Before curing	After curing		
Total	4.70	3.80	4.75	100.0	100.0	-0.90	-19
Protein (insol.)	3.80	1.85	2.31	81	49	-1.95	-51
Soluble	0.90	1.95	2.44	19	51	+1.05	+112
Amino	0.15	0.15	0.19	3.1	4	±0.0	±0
Ammonia plus amide†	0.05	0.80	0.75	1.1	21	+0.75	+1500
Alkaloid	0.40	0.40	0.50	8.4	10	±0.0	±0
Nitrate	0.20	0.25	0.31	4.2	7	+0.05	+25
"Rest"	0.10	0.35	0.44	2.2	9	+0.25	+150

\* Data from analyses carried out by Haley (90), and in the laboratory of *General Cigar Co.*, Lancaster, Pa.

† The actual changes of total ammonia plus amide in the leaves cannot be estimated here because the lost "total nitrogen" consists, not only of ammonia which has been volatilized, but also of an unknown proportion of soluble nitrogenous compounds that migrated from the leaves into the stalk during curing.

The conversions in primed, curing leaves (Table VI) are free of the complication of migration losses. The reasons for the remaining small losses of total nitrogen, which occur as a result of the curing procedure, are not yet fully clear. It is frequently assumed that ammonia or other volatile nitrogenous bases are liberated and disappear from the leaf tissues.

In view of the slightly acid nature of the leaf contents (pH values of aqueous leaf extracts vary between 4.5 and 5.5 at this stage of tobacco treatment), the volatilization of ammonia, etc., can be explained only by the existence of isolated regions of higher alkalinity. Another possibility is (310) a reaction between reduction products of the nitrates (hydroxylamine, nitrous acid) and amino acids which would lead, in the slightly acid tissues, to the liberation of elementary nitrogen. Only a thorough study of the nitrogen balance before and after curing, combined with gas analyses, will provide an answer.

(b) *Leaf Protein and Its Digestion Products.*—The individual elements (amino acids, amides, heterocyclic nitrogen compounds, nucleic acids, etc.)

of tobacco leaf protein have not yet been determined as completely as for some other proteins (see page 319 and reference 193).<sup>\*</sup> But the following facts have been established:

1. About 40 to 50% of the total leaf blade protein is contained in the chloroplasts, with the remainder in other parts of the leaf (86).

2. The nitrogen of the amino acids of leaf protein can be attributed, according to analyses after hydrolysis of the protein (83, 104, 260, 263, 265, 316), to the following groups (in per cent of the total nitrogen of the protein): monoamino, 58%; amide, 7%; basic, 24%; and "rest" (mainly heterocyclic and humin), 11%. The amide nitrogen is represented by the amide groups of glutamine and asparagine, roughly in the ratio of 2.5 to 1 (306, 315, 316). The basic nitrogen must be attributed mainly to arginine and histidine. Both these amino acids have been found in tobacco leaf proteins (160, 317, 354). The "rest" nitrogen contains the heterocyclic nitrogen of tryptophan and proline, together with humin nitrogen. Nucleic acids seem to be present only in small amounts in leaf proteins of healthy tobacco plants (15, 306).

An estimate based on the above approximate figures leads to the conclusion that, of the 58% of amino nitrogen in the protein, 7% is identical with the amino groups of glutamine and asparagine, about 15% with the basic amino acids, arginine, histidine, and lysine, about 4% with the heterocyclic amino acids such as tryptophan, and about 32% with the amino groups of the remaining amino acids present in leaf proteins (such as glycine, alanine, valine, leucine, cystine, phenylalanine, etc.).

3. Apparently, the protein composition changes very little with the type of tobacco. For instance, the results Vickery and Pucher obtained for the leaf of Connecticut shadegrown cigar tobacco are in good agreement, as regards the percentages of amino and amide groups of the protein nitrogen, with the values obtained by Smirnov *et al.* (259, 263, 265) for the leaves of Russian cigarette tobaccos.

4. About 40 to 50% of the protein is converted into soluble nitrogen compounds in the course of curing. The composition of the remaining protein is very similar to that of the original leaf protein. The percentages of amino, amide, basic, and "rest" nitrogen are unchanged except for a slight shift between the amino and the basic nitrogen, in favor of the latter (260, 265, 316, 318).

The unbalanced protein digestion during drying is a process which, under suitable conditions, occurs also in the living plants. Not only do detached

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<sup>\*</sup> This does not refer to the protein of the tobacco mosaic virus, whose composition has been studied extensively (see, *e. g.*, references 206 and 226); the virus protein, however, is quite different from the protein of the healthy plant (15).

tobacco leaves cultured in the dark undergo this conversion, but also leaves of rooted tobacco plants, provided the latter are kept in darkness for several days (162). In the curing process, digestion starts shortly after the leaf has been harvested, and is nearly complete after a few days of curing. The protein left, after 40 to 50% of the original protein has been converted into soluble nitrogen compounds, shows, even after prolonged curing, almost no signs of a further digestion. The first and last of the facts listed above make it likely that this selective response of almost half of the protein is due to its greater accessibility, rather than to a basic chemical difference from the inert part of the protein. The fact that the amount of the inert proteins about equals that of the chloroplast protein, which is protected against the attack of the proteolytic enzymes by surrounding or adsorbed materials, indicates the reasons for the different accessibilities.

Tobacco leaf protein is practically insoluble in water. Inversely, the water-insoluble fraction of the nitrogenous leaf compounds consists almost exclusively of protein. Only small amounts of other nitrogen compounds are present in this fraction, probably decomposition products of chlorophyll belonging to the porphyrins (see page 334). All the other nitrogen-containing substances in the leaf tissues can be extracted with water. Hence, a

TABLE VIII

TRANSFER OF NITROGENOUS GROUPS FROM WATER-INSOLUBLE TO WATER-SOLUBLE FRACTION AND ADDITIONAL CONVERSIONS OF THESE GROUPS DURING CURING OF PRIMED CIGAR TOBACCO LEAVES

Type of nitrogen	Contents, in per cent of dry wt. of uncured leaves				Changes, in per cent of dry wt. of uncured leaves		Needed	Avail- able
	Before curing		After curing		Increase in sol. fraction	Decrease in insol. fraction	In excess of a mere transfer from insol. to sol. fraction	
	Water- sol. part	Water- insol. (prot.) part	Water- sol. part	Water- insol. (prot.) part				
Total	1.92	3.69	3.69 (3.96)*	1.65	2.04	2.04	..	..
Amino	0.23	2.17	0.80	0.85	0.57	1.32	..	0.75
Ammonia plus amide	0.15	0.18	1.07 (1.34)*	0.10	1.19	0.08	1.11	..
Basic "Rest" (hetero- cyclic and hu- min)	..	0.89	...	0.39	..	0.50	..	0.50
	0.56	0.45	0.73	0.31	0.17	0.14	0.03	..
Alkaloid	0.35	..	0.32	..	0.03	..	..	0.03
Nitrate	0.63	..	0.77	..	0.14	..	0.14	..

\* These figures include the total nitrogen lost from the sample during the curing period. The assumption made in including the lost nitrogen in the ammonia-amide group is that it was lost in the form of ammonia from the leaves.



rough idea of protein digestion in the course of curing can be based on the increase of nitrogen in the water extract of the leaf tissues. Table VIII presents a balance sheet for the specific forms of nitrogen which, on the one hand, are set free by the breakup of the proteins, and, on the other, are found as newcomers in the water-soluble nitrogen fraction. The values are based on two thorough experiments by Vickery, Pucher *et al.* with curing Connecticut shadegrown tobacco (310, 318). Values for basic and for "rest" nitrogens of the protein fraction were derived by the writer from the estimated composition of leaf protein (see page 340).

The figures in the last two columns are an indication that the newly formed soluble nitrogen compounds cannot exclusively originate from a mere transfer of each individual nitrogen substance from the protein phase into the soluble phase. A considerable conversion of some protein fragments into other forms must take place to account for the composition of the soluble fraction. The large amounts of ammonia and amides which appear in the soluble fraction are evidently derived both from the amino groups, and the basic nitrogen of the protein fragments. The former change requires an oxidative deamination of amino acids with formation of ammonia; the latter, the hydrolysis (with or without simultaneous oxidation) of the basic nitrogen groups (amino groups other than  $\alpha$ -, and imidazole and guanidine groups) of the digested protein. The ammonia produced by these reactions will form amides without difficulty as long as sufficient amounts of the nonnitrogenous precursor compounds are available for this synthesis. There is no doubt that asparagine is synthesized in this or a similar way in the curing process. Vickery and Pucher (316) suggest that this compound is probably formed, via aspartic acid, by the action of ammonia on fumaric or oxalacetic acids which, in turn, may arise from dehydrogenation of succinic or malic acids. The experimental findings on glutamine as a product of the curing process are the same as those obtained with the leaf cultures in the dark. Whereas glutamine is vigorously synthesized in the assimilating leaf, it does not seem to be formed in metabolism in the dark beyond the amounts performed in the protein fraction.

The formation of amides is an interesting effect because it couples protein digestion with oxidative changes of Dynamic Group substances, in so far as the latter seem to yield the nonnitrogenous reaction partners required for the amide synthesis.

Conversions of the nitrogenous leaf components during curing are essentially the same for most cigarette and cigar tobaccos. Makhorka cigarette tobacco suffers a considerable loss of its relatively large amount of total nitrogen in curing, and the migration effects are very marked in this type of tobacco (150). The change of proteins into soluble compounds, simultaneously with a large increase of ammonia and amide nitrogen, in makhorka, is similar in nature and in extent to the effects in cigar tobacco which Vickery and Pucher have studied so thoroughly. Oriental cigarette tobaccos which, as a rule, are poor in nitrogen (only about 2.3% of the dry weight)

nevertheless show qualitatively the same transformations (40, 104, 105, 263, 265), although much smaller amounts of nitrogen compounds, per dry weight, are involved than in cigar tobacco. These similarities lead one to think that the nature of leaf protein, the system of proteolytic enzymes, and the types of secondary reactions between the split products of protein and other leaf components are common to tobacco leaves of widely different types, a gratifying conclusion, in view of all the variables encountered in this field.

(c) *Nitrates*.—On the whole, the nitrates show no consistent tendency for either a marked increase or decrease as a result of curing. Nevertheless, the nitrate nitrogen is not necessarily inert. Possibly, and even probably, its stability is due to a well-kept balance between its formation from some source, and its disappearance by reduction. The indications for such a role of the nitrates as intermediary products of stationary concentration are:

1. The existence of systematic trends of the nitrate content during curing (310, 318). In some cases, the trend is toward an increase of nitrates, in others, toward a decrease. As a rule, these changes do not exceed a total of  $\pm 25\%$  of the initial nitrate concentration, but they are so definite as to be beyond the possibility of analytical and sampling errors.

The data in Tables VI, VII, and VIII show increases of nitrate nitrogen during curing, but this is not the general rule. There exist also data for a systematic, slow decrease of nitrates in the course of curing. The factors which decide the sense of these trends are as yet unknown.

2. The known, very pronounced effects of nitrates on the metabolism of the living plant, which can be explained only on the basis of a participation of the nitrate group in the reaction cycle in the tissues. This does not necessarily imply a similar reactivity of the nitrates in the starved and dead tissues of the curing leaves, but it makes advisable an even more careful study of the fate of the nitrates during curing before any conclusion as to their passivity is accepted.

(d) *Alkaloids*.—These are present in appreciable quantities in tobacco leaf tissues. Like many *Solanaceae*, the *Nicotianae* contain considerable amounts of these bases. Figure 1 shows the alkaloids which have been identified in tobacco.

The older literature describes tobacco alkaloids which either were not isolated as pure substances or were later recognized as mixtures of some of the compounds listed in Figure 1. N-Methylpyrrolidine and pyrrolidine have also been found, as "alkaloids," in tobacco (276). It is likely that they are split-products of some of the alkaloids in Figure 1.

The names commonly used for these alkaloids are: (1) nornicotine; (2) nicotine; (3) myosmine; (4) N-methylmyosmine; (5) nornicotyrine; (6) nicotyrine; (7) ana-

basine; (8) N-methylanabasine; (9) anatabine; (10) N-methylanatabine; and (11) dipyridyl.

All these compounds are composed of the pyridine ring, to which another heterocyclic ring is attached in the 3-position. In compounds 1 to 6 the heterocyclic ring is a pyrrolidine, a dehydropyrrolidine, and a pyrrole; in compounds 7 to 11, a piperidine, a dehydropiperidine, and pyridine. Compound 2 differs from 1, 4 from 3, etc., by methylation of the nitrogen atom

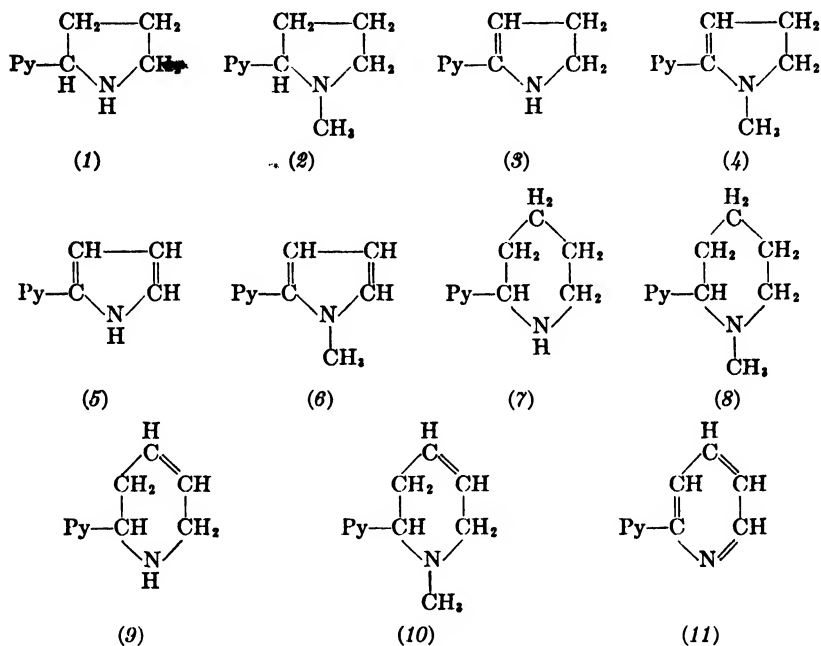
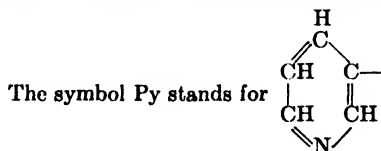


Fig. 1. Alkaloids isolated from tobacco.



of the substituent ring. The composition of these tobacco alkaloids has been investigated by a number of workers (58), particularly Späth *et al.* (276-290) and Wibaut and his school (343-346). Recently, further useful data have been brought to light about the specific reactivities of these alkaloids (23, 70, 89, 222, 300, 350, 351).

As a rule, the *Nicotianae* contain preponderant quantities of one or two alkaloids, and extremely small amounts of the others. It is conceivable that alkaloids 1 to 6, on the one hand, and 7 to 11, on the other hand, are interconvertible within the plant, and that the proportions in which they are found in the leaf tissues conform to their relative stabilities in that environment.

The wild species of the genus *Nicotiana* contain only moderate amounts of alkaloids, the alkaloid nitrogen content ranging from 0.09 to 0.30% of the dry weight, or about 0.5 to 1.8% alkaloid. Four main types of plants can be distinguished, by alkaloid content: (a) nicotine plants (e. g., *N. tabacum*), (b) nicotine plus nornicotine plants, (c) nornicotine plants (e. g., *N. glutinosa*), and (d) anabasine plants (e. g., *N. glauca*) (30, 31, 116, 126, 147, 148, 244, 247, 271-273).

The cultivated strains of *N. tabacum* and of *N. rustica* were probably developed from the wild types of the nicotine, nicotine plus nornicotine, and nornicotine plants. They contain, on an average, two to four times more alkaloids than their ancestors. In selecting and breeding the cultivated strains, desirable properties such as vigor of growth, size of plant, and high content of aromatic substances were aimed at, and it seems that, paralleling the development of these features, the specific productivity for alkaloids automatically increased. Table IX lists some commercial tobacco types and their main alkaloids.

TABLE IX  
ALKALOID CHARACTERISTICS OF VARIOUS COMMERCIAL TOBACCO VARIETIES

Alkaloid	Tobacco		Nico- tine	Norni- cotine	Norni- cotine, per cent in mix- ture	Ref. No.
	Type	Strain	in per cent of dry wt.			
Nornicotine	Cigarette	Robinson Maryland	0.34	1.71	83	(30)
		Medium Broadleaf	0.98	2.16	70	
		Maryland High-Nor- nicotine Tobacco	0.79	1.62	65	(138)
		Cash Flue-Cured	0.70	2.40	77	(30,31)
Mixed (nicotine and nornico- tine)	Cigarette	Burley Halley	1.23	1.41	53	(30)
Nicotine	Cigarette	Turkish	0.71	0.11	16	(148)
		Maryland	1.34	0.29	18	(148)
		Bright	1.72	0.27	13	(148)
		Burley	2.47	0.48	16	(148)
		Maryland Conn. Broadleaf	2.22	0.49	18	(30,31)
		Pennsylvania Seedleaf				
	Cigar	Average crop	2.50	0.30	12	(72)
		Dry-weather crop	3.90	0.48	11	(72)
	Cigarette	Makhorka	4.57	0.99	18	(30)

The percentage of alkaloid increases steadily during growth of the plant. This applies to all its parts, but particularly to the leaves (84, 161, 162, 320). The seed contains no detectable amounts. Traces of alkaloids are found in the seedling as soon as it begins to develop roots, and thereafter the alkaloids increase rapidly during growth, not only in absolute amounts but also in percentages of the dry weight. The leaves of a freshly planted seedling contain, *e. g.*, 0.038% nicotine nitrogen, whereas the leaves of the same plant at maturity contain 0.35% nicotine nitrogen (308, 314).

This increase of alkaloids in the leaves is an uninterrupted, steady process, in contrast to the fluctuations of the other nitrogen compounds (because of digestion, translocation of proteins, etc.) throughout the plant's growth. Day and night, artificial darkness, the changes during flowering and formation of seeds, and other factors which influence considerably the delicate equilibrium of proteins and their digestion products, interfere only minutely, or not at all, with the continuous enrichment of the plant, especially of the leaves, with alkaloids (45, 162-164, 257). This fact alone makes one doubt that nicotine and the related alkaloids are either derived from proteins or participate in the formation of proteins. In the mature plant the highest percentages of alkaloids per dry weight are found in the leaves, with less in the stalk and root. The alkaloids are not distributed uniformly in the leaves, being most abundant at the tips and margins, the sites of most vigorous growth and transpiration. The continuous accumulation of alkaloids in the leaves stops only after they have reached full maturity.

Nicotine in matured tobacco leaves varies from about 0.5 to 7% of the dry weight, with most cigarette tobacco averaging about 1.6% (240, 242), and with cigar tobaccos, about 2.5% (7, 81, 123). Nicotine content of *N. rustica* is considerably higher than that of other strains, running as high as 8%, with a rough average of 4% in mature leaves (74, 131, 146, 270). Fertilization with large amounts of potassium and nitrogen increases the alkaloid content, all other things being equal. Weather, too, has a decided influence: in dry seasons plants contain three to five times more nicotine than plants grown on the same plots with the same fertilization during a wet season (72, 90).

Toth (303) gives nicotine values for dry-weather and wet-weather tobaccos as 5.3 and 2.8%, respectively. Results from the writer's laboratory, on Pennsylvania Seedleaf cigar tobacco, give the following average nicotine values in cured leaves: dry weather (1941), 4.92%; average weather (1939), 2.26%; wet weather (1942), 1.16%. Haley (90) reports the following average nicotine values for uncured Pennsylvania Seedleaf cigar tobacco: dry weather (1943), 5.01%; wet weather (1942), 1.87%.

All these factors formed a complicated picture as long as the view prevailed (1) that alkaloids are formed in the leaves, and (2) that they partici-

pate in the plant's protein metabolism (32). Recent work has led to the astonishing discovery that nicotine is formed exclusively in the roots of the tobacco plant, and from there transported to the leaves by the transpiration stream. [Formation of alkaloids in the root is not a rule for all *Nicotianae*; anabasine is also synthesized in *N. glauca* leaves (48).] Formation of nornicotine has been proved to be of a secondary nature; it originates from nicotine via a demethylation. It would exceed the scope of this survey to describe the great number of experiments (20, 62, 96, 99, 137, 165, 173, 196, 239, 248, 264), particularly Dawson's (43, 44, 46-48) extensive and thorough studies, which led to these discoveries. Suffice it to mention that tobacco scions grafted on tomato roots contain no nicotine in their leaves, whereas the reverse is true of tomato scions grafted on tobacco roots; that nicotine is synthesized from inorganic nitrogen salts in isolated cultures of tobacco root tissue (46); and that its accumulation in the leaves stops abruptly as soon as they have been removed from the stalk (44). The demethylation of nicotine has been proved by experiments in which the leaves of *N. glutinosa*, grafted on roots of *N. tabacum*, formed nornicotine from the nicotine transported from the roots into the leaves of the scion (48).

This demethylation (which may be a transfer of the methyl group to an acceptor, or an oxidative hydrolysis to nornicotine and formaldehyde) has been found to occur in the leaves of nornicotine plants.

The fact that nicotine synthesis takes place only in the roots brings with it the corollary that nicotine accumulation in the leaves is not directly affected by all those factors affecting the parts of the plant above ground.

Certain influences of a secondary order can possibly originate from a mechanism by which carbohydrate formation in the leaves may be linked with nicotine synthesis in the root via a translocation of some carbohydrates into the root, where they might participate in alkaloid synthesis. The possibility of nicotine synthesis in isolated root tissue cultures, however, shows that this effect can be only of an indirect nature.

Nicotine synthesis in the roots also explains the marked dependence of nicotine production on factors controlling the development of the roots (*e. g.*, moisture of the soil and fertilization). As indicated above, the alkaloids are evidently deposited from the current of transpiration at the areas of most intensive transpiration (tips and edges of the leaves); and it is likely that they recede from senescent leaves mainly because they are carried away by the current flowing from these leaves to more vigorous parts of the plant. With this new theory of the formation of alkaloids, the possibility that they participate, as precursors or digestion products, in the protein metabolism of the tobacco leaves is ruled out even more than before.

This conclusion is, however, not to be interpreted as attributing a completely inert role to the alkaloids. There are various indications that the alkaloids, or their transformation products, have a somewhat specific, but nonetheless important, influence on the conversions in leaf tissues, not by participating in these conversions, but by acting as negative or positive catalysts (in the widest sense of this term) for these conversions. Dawson (49) has pointed out that the uptake of nitrates by tobacco plants, and possibly their reduction within the plant, is considerably favored by the presence of nicotine. On the other hand, the oxidative reactions which predominate during the fermentation of tobacco seem to be more retarded the higher the alkaloid content of the leaves (72).

In their structure, the tobacco alkaloids are chemically related to nicotinamide, the prosthetic group of the codehydrogenases (228). Also, they have a tendency to form dehydrogenated derivatives, as shown by the finding of small amounts of myosmine (287) and nicotyrine (331) in the leaves. These facts make it conceivable that the alkaloids, or their transformation products, may exert catalytic influences, as hydrogen donors and acceptors, in oxidation-reduction systems. On the other hand, it is known that primary and secondary amines act as inhibitors of oxidative chain reactions (4, 166), a property by means of which the alkaloids and their derivatives may prevent too rapid oxidations in the leaf tissues. It is thus chemically possible that the alkaloids or their transformation products exert catalytic influences on oxidations or reductions in leaf tissues. This possibility, however, requires further experimental study before definite conclusions can be drawn.

The alkaloids change only slightly during curing (19, 90, 263, 265, 310), showing a net decrease, on an average, of about 3 to 15% of their initial content in the uncured leaves. These decreases, small as compared with the conversions of other nitrogenous compounds, remain about the same whether it is primed leaves or leaves on the stalk that are cured (82). Some observations, which indicate a very small increase of nicotine in primed leaves during the first phases of air curing (318), are in all probability to be explained by the conversion of small amounts of accessory alkaloids into nicotine.

Analytical methods for identification of tobacco alkaloids and their conversion products must be improved and applied to a larger extent before the fate of the alkaloids in the curing leaves can be clarified. Some progress was recently made along this line (72); and the results of these studies will be considered in connection with tobacco fermentation. In this process, the decrease of the alkaloids is very pronounced and constitutes one of the principal conversions in the leaf tissues. At this point it may be noted only briefly that, in all probability, the disappearance of alkaloids in curing is caused not by their volatilization, but by their oxidation to other organic compounds.

In conclusion, it should be added that small amounts of pyridine (27, 202), choline (160, 187), betaine (51), and adenine (354) have been detected in tobacco leaves. Little is known of the origin, fate, or importance of these compounds. Possibly, they are the fragments of alkaloids, amino acids, and nucleic acids, by-products of the hydrolytic and oxidative changes to which the parent substances are subjected.

**Effect on the Dynamic Group.**—(a) *Its Conversions.*—As we have just seen, the tobacco alkaloids tend strongly to keep aloof from the conversions in which many other leaf tissue components are involved, although it is possible that they participate in the control of these conversions via catalytic action. The final resolution of their chemical behavior may therefore be expected to present a comparatively clear and simple picture. This, however, is definitely not true of those tobacco leaf substances which in this report are included in the Dynamic Group (see page 316). This group, composed mainly of carbohydrates, organic hydroxy acids, and unidentified substances of a related nature (unidentified dynamic compounds), is characterized by a high degree of mutability.

The members of the Dynamic Group are very easily transformed one into the other; some of them are speedily synthesized by the living leaf via the assimilation of carbon dioxide; and some are also readily oxidized to the ultimate respiration products, carbon dioxide and water. Exterior conditions and the specific genetic properties of the individual tobacco strains determine which of these processes predominate at a given moment for a given leaf, and, accordingly, the absolute amounts and relative proportions in which the members of the Dynamic Group are present in the leaf. The presence of many fast-acting enzymes causes the dynamic components in the living plant to respond almost instantaneously to even slight changes in light, nutrition, temperature, etc.

During curing and fermentation, *i. e.*, in the starving and dead tissues of the tobacco leaf, these substances still possess a considerable part of their reactivity, although the synthetic processes centered around the photochemical carbon dioxide assimilation stop with the death of the leaves. The chemical elasticity of this group of leaf components explains why their separation and the exploration of their reactions have proceeded only slowly so far. Even if the analytical methods for the determination of the various carbohydrates and related compounds were completely satisfactory—and they are far from being so—a great deal of patient research would still be necessary to resolve into its basic features the complex chemical events in the Dynamic Group.

The difficulties encountered in this group have already been mentioned (see pages 316 and 323). We refer to them here again only to point out that even marked contradictions between the analytical results of various authors in this field are in all likelihood due to differences in the type and history of their samples rather than to inadequate work. Speculations as to the mechanisms of carbohydrate conversions, for example, and similar complicated chemical changes necessarily contain here and there intuitive elements, and are not an unbroken chain of experimentally founded conclusions. This also applies to the following remarks dealing with the fate of the Dynamic Group constituents in the air curing process.

(b) *Carbohydrates.*—The carbohydrate content of harvested leaves varies widely. Some authors report percentages of total carbohydrates as



high as 38% of the dry weight (143, 144), and even 42% for one of its components, starch (292), while others have found values grouped around 3% for cigar tobaccos, and about 23% for cigarette tobaccos (7, 16, 40, 74, 81, 131, 159, 169, 195, 200, 201, 218, 220, 221, 236, 240, 242, 250, 270, 305). The amount of carbohydrates found in a given tobacco leaf removed from the plant depends on many factors: the particular strain of tobacco; the past and present conditions of weather, soil, and fertilization; the hour at which the plant was harvested; the conditions of its storage previous to the analysis; and, finally, the analytical methods employed. As a rule, considerably more carbohydrates are found in cigarette tobaccos than in cigar tobaccos (see Table I, page 313). On the whole, the cigarette tobaccos apparently possess either a stronger capacity for carbohydrate synthesis or a weaker respiratory activity than cigar tobaccos. This physiological difference has probably been developed by the systematic breeding of plants along desired directions; cigarette tobaccos are generally considered to be the better in quality the higher their carbohydrate content, while even high quality cigar tobaccos contain, as finished products, only negligible amounts of carbohydrates. The low-nitrogen fertilizers used with cigarette tobaccos cause a still sharper differentiation between the carbohydrate contents of the two main types of tobacco. The time of harvesting also has the same general aim. Leaves of cigar tobaccos are cut at the peak of their vitality; those of cigarette tobaccos are primed when they have begun to turn yellow (*i. e.*, at the beginning of the senescent stage). In the senescent leaves, the respiratory enzymes are appreciably weakened (see page 352), and the carbohydrates accordingly have a better chance for survival.

Poly-, di-, and monosaccharides are found in tobacco leaves, as in other vegetable materials. Of the polysaccharides, starch has been identified in widely varying amounts.

The high-molecular carbohydrates, cellulose and pentosans, which, because of their stability, are listed in this report in the Static Group, are not considered here. Low-molecular pentoses seem to be present only in very small amounts in tobacco leaves, and only in glucosidic combinations (see rhamnose as a component of rutin, page 335).

In the leaf tissue, the equilibrium between starch and dextrin on the one side, and the soluble carbohydrates on the other, responds almost immediately to demands for storage of carbohydrate material or for withdrawals from these stores. Of the disaccharides, saccharose and maltose have been reported to occur in tobacco leaves (169, 218, 257, 259, 263). Miller (157), by means of a new and interesting method, has identified gentiobiose in the root of the tobacco plant.

Tobacco plants were grown in a medium containing chloral hydrate. This substance is taken up by the plant and forms glucosides with the soluble carbohydrates. The  $\beta$ -glucoside and the gentiobioside of chloral hydrate were found by analysis, their total amounting to 13% of the dry weight.

Apparently, disaccharides accumulate rarely in large amounts in the leaves, but are rapidly hydrolyzed to monosaccharides. Glucose is commonly considered to be the main component among the latter. Studies by Shmuck and co-workers, however, have shown (28, 230, 236) that in certain tobacco types fructose can predominate among the monoses, evidently because of a rapid conversion of glucose into the ketose. No manose has been found in tobacco leaves, but there are indications that other, still unidentified, monoses may be present (309). A considerable part of the glucose is bound in the form of glucosides, a few of which have been identified. [Behrens (16) found glucose to be partly bound on caffeic acid. Rutin is a rhamnoglucoside, and other polyphenols and tannins seem to contain monoses bound with glucosidic linkages.] Galactose, as such, has not been isolated from tobacco leaves, but its oxidation product, galacturonic acid, is the main constituent of pectic acid and the pectins (see page 327). [Large amounts of uronic acids, up to 16.6% of the dry weight, are present in the pith of tobacco stems (168); they probably include glucuronic acid, as well as galacturonic acid. They are present in the form of "polyuronides" (18).]

Many comparative studies of the carbohydrate contents of tobacco leaves include summary analyses, without giving a detailed identification of the individual carbohydrates. Generally, these analyses were based on measurements of the reducing power, with or without a previous removal of phenols and other reducing noncarbohydrate substances. In some instances, differentiations were made between water-soluble and water-insoluble carbohydrates, without and with acid hydrolysis (which splits not only poly- and disaccharides, but also glucosides and related compounds, so that values obtained for total carbohydrates include the glucosidic components), between alcohol-soluble and -insoluble carbohydrates, and between fermentable and nonfermentable carbohydrates (addition of yeast, and determination of the carbon dioxide or alcohol produced) (314, 316, 318). Most authors acknowledge the inadequacy of these methods, which at best give only approximate values. But lacking a better and as simple a procedure, our knowledge of the changes of carbohydrates in tobacco leaves is still almost exclusively based on the summary methods mentioned above.

In the course of air curing, two processes dominate the changes of carbohydrates: (1) the hydrolysis of poly- and disaccharides to monosaccharides, and (2) the oxidation of carbohydrates, via intermediates, to the end products carbon dioxide and water. Obviously, the second process is identical, or at least closely related, to the respiratory process in the living

leaf. Whether there is production of monoses by hydrolysis, or whether they disappear by way of oxidation depends on the type of tobacco and the history of the leaves. Wenusch and Schöller (340) have shown that the vitality of the leaves plays a decisive role in these alternatives.

The green, upper leaves of one tobacco plant lost, in five days of curing, 67% of their total carbohydrates, whereas partly yellow leaves of the same plant, under identical conditions, lost no carbohydrates. In an analogous experiment with another type of tobacco, the vital green leaves lost about 75% of the carbohydrates, in sharp contrast to the partly yellow leaves, which *gained* about 55% of carbohydrates. (This gain is obviously caused by the hydrolysis of nonreducing carbohydrates—starch, etc.—to reducing di- and monosaccharides.) According to the same authors, the respiratory capacity of vital green leaves can be almost completely eliminated by splitting the midrib of these leaves. With three different cigarette tobaccos, the percentage changes of carbohydrates during air curing were as follows: leaves with midribs intact: -90%, -52% -76%; same types of leaves with midribs split: -1%, +39%, -20%. The mechanism of this effect is still not clear, and requires further investigation. Conceivably, the interruption of translocation processes in the leaves by splitting the midribs either prevents the diffusion of respiratory enzymes to their substrates, or causes an abnormal accumulation of inhibitory substances in the tissues.

The enzymic conversion of the poly- and disaccharides to monosaccharides is influenced very little, if at all, by the conditions of curing. Soon after the leaf is detached from the plant, monoses start to appear in increasing amounts, with simultaneous disappearance of equivalent quantities of the higher molecular carbohydrates. But the oxidative conversion of the carbohydrates is slower. In leaves dried very fast at elevated temperatures, the total carbohydrates are conserved almost completely. On the other hand, prolonged air curing, effected by retarded evaporation of the water from the leaves, favors the respiratory consumption of the carbohydrates. As described above, the vitality of the leaves is, other factors being equal, a very important factor in the speed of the oxidative reactions. All cigar tobaccos and several cigarette tobaccos are air cured. In this process, the former, because of the high vitality of their leaves, lose a large portion of their already low carbohydrate content, whether they are cured as single leaves or on the stalk (see Tables IV and V).

In Connecticut shadegrown tobacco the loss of total carbohydrates in curing was found to be about 80% of the initial amount (318).

The cigarette tobacco leaves, especially in the first phase of the curing process, often form additional amounts of sugars from their large stores of starch, etc. (19, 263), but later they, too, lose a part of their carbohydrates. The amount lost depends on the length and conditions of curing. Parallel

with this loss of carbohydrates, the curing leaves absorb oxygen, evolve carbon dioxide, and decrease in weight—all typical signs of the respiratory process (259).

The quantitative effect of curing on conversions in the Dynamic Group is the same as that described for leaves cultured in the dark (see page 321). The total loss of dry weight of the leaves by the end of curing is considerably larger than the total weight of the carbohydrates which have disappeared during this period. In their experiments with curing Connecticut shadegrown tobacco, Vickery and Pucher (314, 316) found weight losses exceeding the detectable losses of carbohydrates by a factor of 4 to 8; for cigarette tobacco, Smirnov found a factor of 2.2.

Calculations of the same type as those given on page 322 prove that the amounts of organic substances which disappear from the tissues during curing in addition to the carbohydrates, are considerably larger than the total losses of all the other analytically determined leaf components.

The conclusion to be drawn is that a large fraction of these losses occurs in the substances included in the Dynamic Group as "unidentified dynamic compounds." Tables IV and V show that the absolute losses of these leaf components in the curing process are greater than those of any other component, and that their relative decreases are of a similar magnitude as that of the carbohydrates.

(c) *Unidentified Compounds.*—The general properties of these compounds can be summarized as follows: (1) Like the carbohydrates, they are formed in the light and oxidized with conversion into carbon dioxide in the dark (see pages 322 and 323). (2) Tobaccos poor in carbohydrates contain greater amounts of unidentified dynamic compounds. Consequently, if a comparison is made on the basis of the sum of the contents of carbohydrates and unidentified dynamic compounds in each type, the marked and "unnatural" differences in the carbohydrate contents of the various tobacco types are greatly diminished. This point, just as the one above, speaks strongly for a very close relationship between the carbohydrates and the unidentified dynamic compounds. (3) According to Neuberg and co-workers (121, 175, 180), the fermentation of tobacco extracts with yeast indicates the presence of fermentable substances which are not carbohydrates. (4) Vickery and Pucher (216), working with Connecticut shadegrown tobacco, and many analyses in this laboratory, as well as by Haley *et al.* (91), on Pennsylvania Seedleaf tobacco, have shown that the sum of inorganic and organic cations in the tobacco leaves exceeds, by a considerable margin, the sum of the anions of the inorganic and ether-

soluble organic acids (citric, malic, oxalic, etc.).\* This discrepancy is only partly removed if the anions of the ether-insoluble organic acids (pectic, resin, phenolic) are added to the total anions. [This statement is based on analyses for pectic, resin, chlorogenic, and quinic acids in Pennsylvania Seedleaf tobacco leaves (72).] (5) Even after prolonged, strong hydrolysis, the unidentified dynamic substances show no signs of the presence of reducing (aldehyde or keto) groups.

This type of hydrolysis (6 *N* sulfuric acid, boiling, 20 hrs.) suffices to hydrolyze all the polysaccharides and glucosides contained in the tissues, or in extracts, to the corresponding reducing sugars. The abnormally high values found by some authors for pectins (see page 328) possibly include some of the unidentified dynamic compounds.

In the writer's opinion, these properties of the unidentified dynamic compounds support the concept that a large part of them is identical with, or closely related to, simple oxidation products of the monoses, in which the characteristic reducing group of the sugars has been converted to a carboxyl group, *i. e.*, with the formation of substances of the type of hexonic (gluconic) acids. It is known that gluconic and related acids are easily formed *in vitro* (71), as well as by the action of microorganisms and enzymes (see page 355) as products of carbohydrate oxidation. These acids are yeast fermentable. Their presence in quantities of several per cent would furnish enough acidic anions to satisfy the demand of electroneutrality, and their extremely low solubility in ether and many other organic solvents would explain why they are not found among the "total organic acids."

This term is, unfortunately, widely used for the special fraction of organic acids which can be extracted by organic solvents from acidified tobacco tissues or tobacco extracts. A considerable number of organic acids which are soluble in water only is not included in this fraction.

The soundness of this speculation can only be decided by the analytical detection and quantitative determination of the unidentified dynamic compounds.

Experiments under way in this laboratory indicate that the barium and lead precipitates obtainable from aqueous tobacco extracts freed of all volatile bases yield, on decomposition and after removal of the barium and lead, an acid fraction containing, besides the acids known to be in tobacco, further amounts of alcohol-insoluble acids which are not pectic acids.

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\*This is true even if a full electrochemical neutralization of the 2- and 3-basic organic acids is postulated, a demand which is unjustified in view of the fact that the average *pH* of tobacco extracts (4.5 to 6.8) indicates an only partial neutralization of the acids. Hence, the lack of analytically determined acids is even greater.

Vickery and Pucher (316, 319) offer another hypothesis concerning the nature of the substances lost from the leaf tissues in the curing process (and during dark culture of detached leaves), in excess of the disappearing carbohydrates. Their theory is as follows: A part of the amino acids which are formed as digestion products of the leaf protein is converted, by oxidative deamination, to ammonia and nonnitrogenous organic (hydroxy) acids (page 342); the latter are possibly identical with the substances which, in addition to the carbohydrates, are oxidized to carbon dioxide. Quantitatively, the amounts of these deaminized acids would suffice to account for the excessive weight losses.

There is no doubt that processes of this kind do contribute to the "total respiration" of the tissues, but, in the writer's opinion they are only of a subordinate character as compared with the oxidative consumption of substances more closely related to, and directly derived from, the carbohydrates. This view is supported by observations (1), (2), and (3) (page 353), as well as by the fact that further losses of the dry weight occur in the fermentation of tobacco without any appreciable losses of carbohydrates, or simultaneous digestion of proteins. It is, however, interesting that both the "gluconic-acid hypothesis" and the theory of Vickery and Pucher attribute the excess respiration of tobacco leaves to the oxidation of water-soluble, ether-insoluble organic acids.

Proof of the existence of appreciable amounts of hexonic acids in the leaf tissues would strongly indicate that the oxidative degradation of the carbohydrates proceeds, to a considerable extent, via these acids, and not, as is widely postulated, by the splitting of one hexose molecule into two triose units, as the initial step of carbohydrate oxidation.

The existence of considerable amounts of uronic acids in the leaves and stems of the tobacco plant shows that an oxidation of carbohydrates is possible, without a break of the C<sub>6</sub>-chain. The enzymic oxidation of glucose (mannose, galactose) to the corresponding hexonic acids is well known. Several microorganisms, such as *Aspergillus niger* and *Penicillium glaucum*, contain enzymes which act specifically in this direction (21, 167). Some *Fusaria* have been found to oxidize glucose (184) and pentoses to their corresponding nonreducing acids (97). Specific dehydrogenases have been isolated from liver preparations by Harrison (94). In combination with an oxygen activator (such as indophenol oxidase) and an oxygen carrier (such as cytochrome); they form gluconic acid from glucose. Stotz (293) has recently pointed out that a number of facts support the possibility of a direct oxidation of glucose, without previous splitting of the molecule, as an alternative first step in the mechanism of respiration, even in animal tissues, and Ochoa (189) gives a more detailed analysis of this system. Thus, the assumption of gluconic acid as the initial oxidation product of carbohydrates in tobacco leaves can be used as a working hypothesis, without conflicting with facts from the enzymic point of view.

Seen from this angle, the existence of particularly large amounts of unidentified dynamic compounds in the vital cigar tobacco leaves would indi-

cate that they contain a more active enzyme system for the conversion of carbohydrates to hexonic acids than the relatively senescent cigarette tobacco leaves. The decrease of the unidentified dynamic compounds, during curing, with the simultaneous evolution of carbon dioxide and considerable loss of dry weight, would correspond to the further oxidative reactions which lead, over a series of intermediates, from hexonic acids to carbon dioxide (and water) as the end product. It is known, in principle, that gluconic acid can be oxidized to various degradation products (152). However, the specific path of reactions over which oxidation of the carbohydrates is affected in the tobacco leaves has not yet been established with certainty. There are, nevertheless, several indications that hydroxy and keto acids play an important role as intermediates in this process.

(d) *Ether-Soluble Organic Acids*.—These are only a fraction of the total acidic organic compounds in the leaf. Pectic, resin, phenolic, and oxalic acids have already been described as members of the Static Group. Amino acids, the building blocks of tobacco proteins, appear among digestion products of proteins. There are indications of the presence of further nitrogen-containing acids in the leaf tissues. [Vickery and Pucher (313) obtained from a barium precipitation of tobacco extract certain acids which were insoluble in ether. After determination of the amino acids in this fraction, a proportion was left which still contained nitrogen and possessed an acidic character. Some data on the nature of these substances, based on results obtained in this laboratory, will be given in the discussion of tobacco fermentation.] Finally, the possibility that hexonic acids are important components of the Dynamic Group has just been discussed.

The principal representatives of the ether-soluble acids isolated from tobacco leaves are malic and citric acids, amounting together to about 11% of the dry weight. In addition, very small amounts (1 to 2% of the dry weight) of other ether-soluble acids are present, only a few of which have been identified: formic, acetic, succinic, and fumaric acids. Pucher and Vickery (210, 211) found an average of 0.02 to 0.05% each of succinic and fumaric acids, and Garner (81) reports 0.1% as an average for formic acid. The remaining unidentified ether-soluble acids are obviously mixtures of a number of organic acids, probably of more complex structure.

A significant feature of the citric and malic acids occurring in the leaf tissues is their tendency to replace each other during growth of the plant and treatment of harvested leaves. As a rule, malic acid predominates in the young leaves; citric acid increases up to the plant's maturity, with a simultaneous decrease of malic acid (32, 118, 266). The relative proportions of the two acids, like the carbohydrate content, respond very quickly

to factors such as growth, weather, nutrition, and conditions of treatment of harvested leaves. A greater uniformity, however, prevails for the malic-citric combination than for the carbohydrates, since their sum remains fairly constant. This is true both of the malic-citric content of different tobaccos and of any one tobacco, during its later growth and curing. The sum amounts, on an average, to about 9 to 14% of the dry weight, for both cigar and cigarette tobaccos. In the curing process, these total values decrease slightly to about 8 to 12% of the dry weight (see Tables IV, V, and XI).

The stability of the sum of the two acids, together with the fact that their equivalent weights, 64 and 67, are of a similar magnitude, results in a corresponding stability of the acidity\* represented by these two main components of the ether-soluble acids. In contrast, the ratio of the two acids shows very pronounced changes. In the living leaf, citric acid forms at night at the expense of malic acid (324). Furthermore, all the factors favoring increase of carbohydrates also favor, as a rule, shifts between the acids in the direction of increased citric acid content. This is more obvious in cigar than in cigarette tobaccos. In the latter, the effect seems to depend on the additional factors controlling the oxidation or conservation of carbohydrates (322): the higher the tendency toward oxidation, the more pronounced the shift toward citric acid. It is concluded from these and other observations that (1) malic and citric acids are both involved in the cycle of reactions which comprise carbohydrate oxidation, and (2) in this cycle, citric acid represents an intermediate closer to the final stage of complete oxidation than malic acid.

The much more consistent replacement of malic by citric acid in cigar than in cigarette tobaccos is obviously connected with the faster and more extensive conversion of the carbohydrates to their first oxidation products (hexonic acids?) in the former. When this first step is retarded, or is on a small scale—probably the case in many types of cigarette tobacco—the opportunities for the succeeding oxidative steps are bound to be limited. This also explains the inconsistent results of experiments in which glucose was injected into cigarette tobacco leaves in order to see whether increased amounts of citric acid would form. Several authors found indications of a chemogenetic link between glucose and citric acid (274), but it was not confirmed by others (88, 156, 204, 205), who found, instead, that formation of citric acid increased strongly only after infiltration of malate, fumarate, oxalacetate, and succinate. The present concept of the mechanism of carbohydrate oxidation and citric acid formation, discussed below, explains these results to a certain extent.

The ratio of malic to citric acid ranges from about 9:1 to 1:10, depending on whether reductions or oxidations predominate at the time of sampling.

\* This term does not mean "free acidity" but rather "equivalent value,"

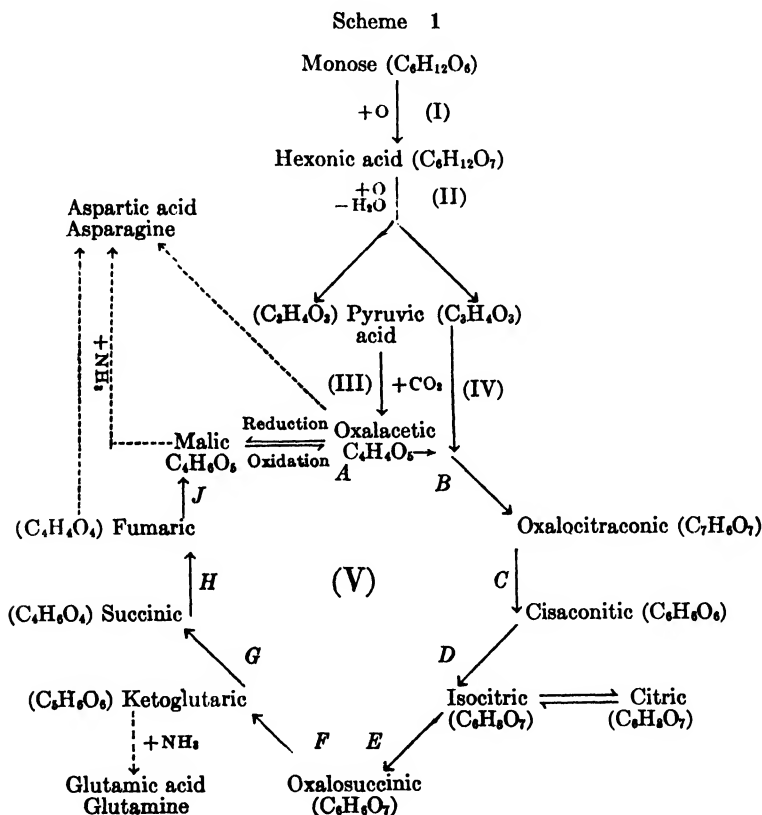


Normally developed leaves contain, on an average, about 6.7% malic and 3.7% citric acid (81, 118, 231). This ratio was also found in the leaves of thirteen wild *Nicotiana* species (115). However, appreciably greater amounts of malic acid have been found in several instances, with values of 11% for malic and 0.5 to 1% for citric acid (231, 311–313), while others (90) have found amounts between these limits. But in all cases the sums of both acids remain nearly constant between 9 and 14% (32, 40, 87).

The predominance of the oxidative conversions in the curing process results in marked shifts in favor of citric acid. Vickery and Pucher, following this conversion both in the curing process (311, 313) and in dark cultures of detached leaves (316), found that, in curing, the ratio of malic to citric acid changed from about 10, at the start, to about 2 after twelve days, and to about 1.4 for the fully cured leaves. In leaves cultured in the dark, the ratio changed for the entire period from 4.5 to about 0.5. In Pennsylvania Seedleaf tobacco Haley found curing caused a change in the ratio from 2.7 to 0.4 in wet-season tobaccos, and from 2.1 to 1.0 in dry-season tobacco. Here again, the total acidity remained practically unchanged, despite the considerable internal conversions. While these data indicate good reproducibility of the shift effect in cigar tobaccos during curing, no similar uniformity is revealed by analyses made of curing cigarette tobaccos. Here, as a rule, the shift effect is smaller and varies considerably with the age of the leaves, type of tobacco, etc. (34, 231, 256, 316). Furthermore, the total acidity represented by the ether-soluble organic acids does not remain at such a constant level during drying in the cigarette as in the cigar tobaccos. The total acidity increases or decreases by 10 to 40%, depending on the type of tobacco and history of the samples. This irregular response to curing of the ether-soluble acids in cigarette tobaccos is also probably the result of the changing capacity of these tobaccos for the primary oxidative attack on the carbohydrates.

(e) *Mechanism of Carbohydrate Oxidation*.—Several authors, particularly Vickery, Pucher *et al.* (316, 319), have exhaustively discussed the possibilities of a chemical mechanism which is in accord with all these observations. Obviously, many theories fulfill this condition. In order to base speculations of this kind on a broader experimental foundation, one must determine whether citric and malic acids, as intermediates of carbohydrate oxidation, or of respiration generally, occur in other biochemical systems. It has been observed that citric and malic acids exert a strongly accelerating action on the respiration of animal tissues. Vickery and Pucher have reviewed the experimental findings on this subject of Szent-Györgyi, Krebs, Knoop and Martius, Chibnall, and others, and discussed

its implications for carbohydrate oxidation in tobacco and rhubarb leaves (319). In addition, valuable surveys have recently appeared on the present state of these theories (128, 293), so that only the assumed mechanism of the citric acid cycle in the tobacco leaf, modified to fit the theory of the first oxidative change of the carbohydrates (page 354), is given here. Scheme 1 illustrates the course of the reactions.



The fate of the monose, the substrate of the general oxidative process, can be divided into the four main phases as shown in Scheme 1: (I) transformation into hexonic acid; (II) conversion of hexonic acid into two molecules of pyruvic acid; (III) and (IV) entrance of pyruvic acid into the cycle; (VA) to (VJ) the cycle proper which is equivalent to the complete oxidation of one molecule of pyruvic acid to carbon dioxide and water. Steps (I) and (II) differ from the usual scheme (monose  $\rightarrow$  2 trioses  $\rightarrow$  2

pyruvic acid molecules) only by the immediate oxidation of the monose to hexonic acid. The reasons for introducing this specific reaction step have already been discussed (see page 353). The accumulation of carbohydrates in some cigarette tobaccos is, according to this idea, due mainly to the slowness of step (I).

Pyruvic acid is now recognized as one of the key substances in carbohydrate fermentation and oxidation (185, 293), and its conversion into oxalacetic acid, by taking up carbon dioxide, was recently demonstrated experimentally (129, 347). Similarly, the condensation of pyruvic acid with oxalacetic acid to oxalocitraconic acid has been postulated on the basis of experimental data (348). An important feature of cycle (V) seems to be the oxidation-reduction equilibrium (VA) between malic and oxalacetic acids. In a reducing environment, as in the presence of a large excess of carbohydrates (slowness of I), or during the synthesis of reducing compounds via photochemical assimilation, the equilibrium can be expected to shift largely in favor of the malic acid, and only a negligible part of the over-all reaction will follow the clockwise oxidative scheme  $B \rightarrow C \rightarrow D$  which leads to the citric acid. The conditions in many cigarette tobacco leaves probably favor this state of preferred malic acid content. On the other hand, the disappearance of reducing substances such as carbohydrates, phenols, etc., during the particularly active curing of cigar tobacco favors the clockwise scheme of oxidative reactions, so that they soon control the over-all conversion, with complete oxidation of parts of the original carbohydrates. In the scheme outlined in Scheme 1, citric acid is located in a "switch" position; it will accumulate, provided the speed of isomerization of isocitric acid to citric acid is appreciably greater (for comparable concentrations) than the reverse reaction. The other compounds, at the successive stages of the cycle, will accumulate in amounts controlled by the relative speeds of their formation and disappearance. Apparently, most of these intermediates are converted so speedily that their stationary concentrations are kept at a low level. Nevertheless, the presence of appreciable quantities of unidentified ether-soluble acids in tobacco leaves may indicate that, besides pyruvic acid, other members of cycle (V) are contained in this fraction.

When Scheme 1 is tabulated (Table X), it becomes clear that the total effect of one full rotation through the cycle is the complete oxidation of one pyruvic acid molecule to carbon dioxide and water. This oxidation is accomplished by linking this pyruvic acid molecule to another pyruvic acid molecule ( $+ \text{CO}_2$ ), with the formation of more complex molecules. This "carrier principle" evidently leads to a faster oxidation rate than the direct oxidation of a single pyruvic acid molecule. It is known (73) that the reactivity of chemical groups can be materially enhanced by their attachment to more complex molecules. [This is due to a decreased energy of activation for the reactive step and to an increased probability that this energy of activation is accumulated, in a certain period of time, in the reacting bond (see reference 73).] Malic and citric acids are obviously the most stable compounds of the cycle, and they apparently accumulate to a sufficient extent to establish a certain pH in the tissues. Possibly this effect

TABLE X

REACTION STEPS OF THE CITRIC ACID CYCLE AND PRECEDING OXIDATIONS\*

Step No.	Nonoxidative steps			Oxidative steps		
	Decarboxylation	Dehydration	Hydration	CO <sub>2</sub> formed by oxidation of CO of substrate	H <sub>2</sub> O formed by oxidation of H of substrate	Atoms oxygen required
	-CO <sub>2</sub>	-H <sub>2</sub> O	+H <sub>2</sub> O	-CO <sub>2</sub>	-H <sub>2</sub> O	+O
VA	...	...	...	...	1	1
VB	...	1	...	...	...	...
VC	...	...	...	1	...	1
VD	...	...	1	...	...	...
VE	...	...	...	...	1	1
VF	...	...	...	1	...	1
VG	1	...	...	...	...	...
VH	...	...	...	...	1	1
VJ	...	...	1	...	...	...
Balance	-1	-1	+2	-2	-3	+5

\* The step numbers refer to Scheme 1. H<sub>2</sub>O and O which go "into the cycle" are listed as +H<sub>2</sub>O and as +O. H<sub>2</sub>O and CO<sub>2</sub> which go "out of the cycle" are listed as -H<sub>2</sub>O and as -O. Thus 5 O "go into the cycle," with the formation of 3 CO<sub>2</sub>, and 2 H<sub>2</sub>O go "out of the cycle." In other words, C<sub>3</sub>H<sub>4</sub>O<sub>3</sub>, or one molecule of pyruvic acid, is completely oxidized.

is caused by an automatic self-regulatory mechanism by which formation of the acids (or, better, the enzyme system which catalyzes their formation) is favored by pH values on the active side up to a certain limit, after which the reaction comes to a standstill.

The connection between amino acid metabolism and carbohydrate respiration was mentioned on page 355. Three potential precursors of aspartic acid and asparagine are represented in the citric acid cycle (Scheme 1), and one precursor of glutamic acid and glutamine. It is not clear why, in tobacco leaves, glutamine is synthesized in the light (see page 342), but not in the dark, since the respiration cycle offers ketoglutaric acid as the presumptive precursor in both cases. As a mere speculation *ad hoc*, the possibility might be mentioned that the dehydration + aminization, or the direct transamination, of ketoglutaric acid is a photochemical process. We refrain from discussing here whether or not phosphorylations are important and necessary within the frame of the proposed scheme of oxidation, and also from going into the details of the single steps of the cycle. This has been thoroughly done by other authors, and there are at present no indications that the study of the processes in the tobacco leaf will contribute new ideas to these questions.

In concluding this section, the writer wishes to emphasize that the above interpretation of the carbohydrate metabolism of the tobacco leaf is by no means the only possible working hypothesis in accord with the analytical results obtained. To quote Vickery (319): "It is at once the merit and

the deficiency of such schemes that an *experimentum crucis* is almost impossible to devise."

### 3. Flue Curing

Some of the chemical processes which occur in tobacco leaves during air curing are very rapid conversions, *e. g.*, the digestion of considerable amounts of leaf proteins, the breakdown of polysaccharides into simpler sugars, and, to a limited extent, the respiratory oxidation of a fraction of these sugars. Significantly, these reactions are continuations of metabolic processes in the living leaf. Others occur more slowly, such as the transformation of chlorophyll, the changes of pectins, and the various conversions involving resins and volatile oils. The slowest reactions are: (1) oxidations in which the remaining carbohydrates are attacked, with the formation of various intermediates such as hydroxy acids; (2) oxidations in which split products of the proteins are deaminized, with the formation of ammonia and amides; and (3) oxidations in which phenolic compounds are transformed into dark-colored products.

This separation into three sets of reactions, in accordance with their relative speeds, of course does not imply that there exist three corresponding, sharply demarcated periods in the air-curing process. Actually, the various conversions tend to overlap each other. Nevertheless, one after the other, each of the three sets of chemical processes becomes the dominant one during each succeeding stage of air curing.

The different chemical transformations are, also, selectively dependent on the water content of the curing leaves and on temperature. As mentioned before, the chemical composition of the leaves can be "frozen" at any point by a fast dehydration of the tissues. Thus, rapid desiccation of the leaves in a vacuum will conserve almost all the carbohydrates, the chlorophyll, large quantities of the proteins, the phenols, etc. The enzymes evidently require a certain amount of water in the leaf tissues to carry on their specific activities. Remoistening leaves which were desiccated at normal temperatures revives most of the chemical processes of air curing. High temperatures, however, cause not only rapid dehydration of the leaves but also permanently inactivate some of the enzymes, thus producing irreversible changes. Some enzymes, for example, the carbohydrases, are comparatively little affected by higher temperatures (see page 367), whereas many of the oxygen-transferring enzymes are destroyed by heat.

The difference in the reaction speeds and their dependence on water content and temperature are the basis of flue curing.

In the United States, several cigarette tobaccos, mainly those grown on light and sandy soils in Virginia, North and South Carolina, Georgia, Florida, and to some extent in Alabama (77), are flue cured. They differ from all cigar tobaccos and from most other cigarette tobaccos by their low nitrogen and ash contents especially, and by their high carbohydrate content. Furthermore, they apparently have more volatile oils and less resins ("gums") than most of the other tobaccos.

In flue curing, the tobacco leaves are cured in closed barns. In some cases, the whole plant is harvested and leaves are cured on the stalk, but more usually the leaves are primed. The barns are heated by means of sheet-iron flues which rest on the floor, and which are connected with a stove outside. The heat is administered in three separate periods, approximately as follows (25, 36, 39, 55, 78):

Period	Temperature, ° C.	Duration
Yellow or color-setting	32° to 37°	24 to 36 hrs.
Fixing	54° to 60°	10 to 24 hrs.
Killing	80° to 90°	0.5 to 3 days

In the first period, chlorophyll is converted into colorless or yellowish products, and the pure yellow colors of carotene and xanthophyll become dominant. In the second period (fixing), the leaf blades are completely desiccated but the stems still remain fairly pliable; the color turns somewhat duller. In the third period, the stems have dried and are very brittle. Normal temperature is then restored and humid air admitted. After some time, the leaves have re-adsorbed enough moisture to permit their being handled without breakage.

Table XI shows the constituents of cigarette tobacco leaves before and after flue curing. The cigarette tobaccos which are flue cured are tobaccos with abundance of carbohydrates and a comparative lack of nitrogenous compounds even in their green state. Flue curing increases this difference from the typical cigar tobaccos and from most other cigarette tobaccos even more sharply. The most conspicuous effect of flue curing, contrary to that of air curing, is the preservation of large amounts of carbohydrates. Frequently, there even occurs an increase of the soluble disaccharides and monoses (due to the transformation of starch into the simpler sugars). Other signs of the less extensive oxidation in flue curing are the smaller amounts and the slighter decreases of the unidentified dynamic compounds, and the smaller losses of dry weight. Suppression of the oxidative reactions is further indicated by the higher ratios of malic acid to citric acid in the flue-cured leaves, by the much smaller amounts of ammonia (fewer

TABLE XI  
CHEMICAL CHANGES IN PRIMED, FLUE-CURED CIGARETTE TOBACCO LEAVES

Constituent	Amt. in per cent of dry wt.		Changes, in per cent of		Amt. in per cent of dry wt. of cured leaves
	before curing	after curing	harvested dry wt.	initial amt. of constituent	
Ash (inorg. cations and anions)	12.0	12.0	0	0	13.5
Crude fiber (cellulose and lignin)	10.0	10.0	0	0	11.2
Pentosans	2.0	2.0	0	0	2.2
Pectins	7.0	7.0	0	0	8.0
Ether-sol. compds. & sol. oils, resins, waxes, paraffins)	7.5	6.5	-1	-16	7.3
Tannins (polyphenols, phenolic acids)	2.0	2.0	0	0	2.2
Oxalic acid	2.0	2.0	0	0	2.2
Nitrogenous compds. (proteins, amino acids, amides, ammonia, nitrates, alkaloids)	15.5	13.5	-2	-12	15.2
Total nitrogen	2.7	..	..	..	2.6
Carbohydrates (poly- and mono-saccharides, starch and dextrin)	23.0	20.0	-3	-13	22.5
Ether-sol. org. acids (citric, malic, unidentified)	11.0	9.0	-2	-18	10.1
Unidentified dynamic compds.	8.0	5.0	-3	-36	5.6
<i>Total</i>	100.0	89.0	-11	..	100.0

oxidative decompositions of amino acids, etc.) (39-41), by the preservation of larger amounts of volatile oils (less resinification), and by the "freezing" of the phenolic compounds present in the green leaf. Rutin, for instance, is still found in appreciable quantities in flue-cured tobaccos (37, 38, 177), whereas it completely disappears from the air-cured ones. The preservation of the yellow color in flue-cured tobacco is an external sign that the reactions in which polyphenols and tannins are converted into dark brown oxidation products have been suppressed. Of the nonoxidative reactions, the enzymic demethylation of the pectins is at a considerably lower level in flue curing. Both rapid loss of water and the destruction of the more labile enzyme systems at the high temperatures employed seem to be the causes of all these features of the flue-curing process.

Characteristics common to both air and flue curing are: (1) the chemical stability of cellulose, lignin, oxalic acid, and of a large proportion of the ether-soluble compounds; (2) the decomposition of leaf proteins to simpler nitrogen compounds; (3) the hydrolysis of polysaccharides to di- and mono-saccharides.

In its final outcome, flue curing results in tobaccos which produce an acidic smoke of a very light aroma, because of their high carbohydrate and

low nitrogen contents. Air curing, however, particularly of the cigar tobaccos, results in tobaccos which produce an alkaline smoke of a fuller aroma (333, 335).

#### 4. *Fire Curing*

As far as can be ascertained, there are no detailed studies of the chemical processes which occur in fire curing, the only exception being a study by Splendore (291) on fire-cured Italian tobaccos.

In the United States, fire curing is used in parts of Virginia, Kentucky, and Tennessee for a few special cigarette tobaccos which have heavy, dark green very gummy leaves. A similar process is used in Latakia (in the Levant), for curing small leaves of Turkish cigarette tobacco (77, 171). Only a fraction of the fire-cured tobacco is used for smoking purposes, the largest part going into the manufacture of snuff.

As in flue curing, the tobacco is subjected to high temperatures, although they are not always as high as those in the third phase of flue curing. The heat is generated by open wood or charcoal fires in the closed barns, and the leaves hang, densely crowded, over the fires. An additional effect of fire curing is that some of the smoke constituents are deposited on the surface of the leaves. Naghski, Beinhart, and Couch (171), who studied the behavior of fire-cured tobaccos during fermentation, report that the phenol content of fire-cured leaves is fifteen times greater than that of comparable air-cured samples (0.316 as against 0.021%), and some Latakia samples even averaged 0.626% of volatile phenols. Obviously, this increase in phenol content arises from the smoke to which the leaves were exposed.

### IV. *Enzymic Processes in Tobacco Curing*

#### 1. *Enzymic Conversions in the Leaves*

In many respects, the curing of tobacco is equivalent to a continuation and intensification of some of the chemical processes in the living plant. Vickery and Pucher (316) have shown that metabolism of detached, cultured tobacco leaves in the dark is very similar to the reactions during the starvation period of air curing. After the leaf dies, the gravely disturbed biochemical equilibrium in the leaf is shifted still further toward the decomposition and oxidative conversion of its components. There are no indications that any chemical process in the leaf cannot be attributed to the action of enzymes which are, *a priori*, contained in its tissues. Hence, it is generally acknowledged that external agents, such as bacteria or molds, play no important role in the drying or curing of tobacco. However, the



action of microorganisms is considered, by some authors, as necessary and decisive for the *fermentation* of tobacco.

This does not preclude the fact that, under unfavorable conditions, many different bacteria and molds can attack tobacco also during the drying process. Such effects, however, are undesirable accidents, and are prevented by avoiding excessive moisture and stagnant air, particularly in that phase of curing which follows the death of the leaves, and for crops which grow in a wet season.

## 2. *Tobacco Leaf Enzymes and Their Role in Curing*

Many enzymes and enzyme complexes have been reported to be present in tobacco leaves. However, there are no observations that indicate a fundamental difference in the nature and activity of enzymes between tobacco and other plants. Although the material dealing with tobacco enzymes is prolific, only little of it contains facts which help to clarify and extend our present knowledge of the driving factors behind the various chemical changes described here. The reasons for this slow advance lie, just as in the purely chemical research on tobacco, in the great variety of tobaccos studied, and the frequent use of methods which are not comparable.

Tests for the presence of various enzymes in tobacco leaves are commonly carried out by adding a tobacco extract to a reaction system which responds specifically to an enzyme whose presence in the leaf extract is suspected. Only in a few instances have investigators attempted and achieved an enrichment of the enzyme in the leaf extract by removing accessory inert or inhibiting substances in the extract. It is obvious that tests of this kind can give only approximate information. In some cases, in order to obtain a more complete characterization of the enzyme, more detailed studies have been made of the relation of enzyme activity to the pH, temperature, and concentration of the substrate. In several instances in which the presence of oxidizing enzymes have been reported in tobacco extracts, the experimental observations can be equally well interpreted by the assumption that not specific enzymes but autoxidizable compounds, together with simple organic or inorganic catalysts, have caused these effects.

The following brief review of the tobacco leaf enzymes is limited to the specific types that have been identified.

### HYDROLASES

The living plant can store carbohydrates and nitrogenous substances as chemically inert reserve materials, and retransform them quickly into mobile and reactive compounds whenever the need arises. Specific, fast-acting enzymes are responsible for these rapid changes.

(a) **Carbohydases.**—The almost instantaneous response of the carbohydrate complex to the changes incident to the plant's development

and to external influences is caused by the carbohydrases, which act as catalysts in establishing equilibrium between the poly-, di-, and monosaccharides. In the living plant this equilibrium is in a continuous state of delicate balance, with the enzymes catalyzing both the synthesis and breakdown of the higher molecular carbohydrates (251). [For the synthetic action of invertase and peptidases in the living leaves, see (136).] But after the leaves are harvested, the breakdown of the more complex compounds to smaller units prevails over the synthetic reactions.

This is not the case for all the chemical reactions which occur during the curing of tobacco leaves. Higher molecular resins are partly formed from lower molecular compounds, and phenols are condensed, under simultaneous oxidation, to tannin- and melanin-like materials. During fermentation, a considerable amount of proteins, or at least of protein-like substances, is formed from simpler nitrogen compounds (72).

*Amylase*.—The starch-hydrolyzing activity of tobacco extracts is very pronounced (1, 8, 82, 142, 159, 190, 194, 255, 263, 266, 275, 353). According to Neuberg and Kobel (175, 176), a macerate of 200 g. green tobacco leaves completely hydrolyzes 20 g. soluble starch in three days. A number of authors (149, 191) have found that the pH optimum of tobacco amylase is between 6 and 7.4, and its temperature optimum is at about 40° C. A significant property of the amylase in tobacco leaves is the increase of its activity during curing. Smirnov and Drobglav (263) found an approximately 20-fold increase in the starch-hydrolyzing capacities of the extracts of unripe leaves, and about a 4- to 5-fold increase of the extracts of ripe, cured leaves. Similar effects are reported by Garner *et al.* (82), Smirnov (255, 266), and Kimpoy and Matsushima (117). Whether this effect is due to a liberation of previously bound enzymes, to a synthesis of the enzyme, to the formation of activators, or to the disappearance of inhibitors, is still undecided. Neuberg and Kobel (176), as well as Volgunov (325), found that even after drying fresh leaves for 1 hr. at 100° C., the amylase activity was only slightly diminished.

*Sucrase (Invertase,  $\beta$ -*h*-Fructosidase)*.—This tobacco leaf enzyme has been studied by most of the authors who investigated the amylase, as well as in some special experiments (127, 152, 297, 326, 341). Its activity increases moderately in mature leaves in the course of curing (+40%), and decreases slightly in unripe leaves (−15%) (263). This enzyme, too, shows no considerable decrease of its activity after a short treatment of the leaves at 100° C. (This property of carbohydrases seems not to be restricted to tobacco leaves. Platonenko (207) found that the sucrase activity of geranium leaves was highest after drying at 85° C., and was completely

destroyed only after drying at 150° C.) Its pH optimum is 4.5 to 5 (149, 192, 260), and its temperature optimum, 45° C.

In the living plant, amylase and sucrase accumulate in the tips of the leaves (353); the branches, ribs, and stems, in the order named, contain decreasing amounts (341). The hydrolysis of sucrose in tobacco leaves is not prevented by the presence of toluene and chloroform. Very low moisture content of the leaves and of the surrounding air also has no retarding effect, a proof that microorganisms play no role in this conversion (256).

Of other carbohydrases, *inulase* has been found in tobacco leaves (194). It is possibly identical with sucrase. *Maltase* ( $\alpha$ -glucosidase), reported as a component of freshly harvested tobacco leaves by Volgunov *et al.* (328), was not detectable after curing. The same authors claim that fresh mature tobacco leaves contain no  $\beta$ -glucosidase (*emulsin*), a statement in conflict with the results of previous investigators (1, 17, 175, 181, 182, 194, 260) who found glucoside-splitting enzymes of the emulsin type in harvested leaves, and an increase by 10 to 100% as a result of curing. Obviously, some glucosidase activity must be present, or must be generated, during the drying process, in view of the decomposition of rutin and other glucosides during air curing.

(b) **Esterases.**—The pectin-splitting enzymes of tobacco have been studied particularly by Neuberg and his school (175–182), as well as by others (53, 114). About 50% of the methyl alcohol bound in the pectin is split off in 2 hrs., and about 90% in 5 weeks, by the action of the pectase in leaf tissue during autolysis of macerates of green tobacco leaves. In similar experiments by Kertesz (114), 33% demethylation of the pectin occurred, at pH 6.2 in 0.5 hr., and complete demethylation in 46 hrs. The mixture used was one part tobacco extract, two parts of a 1% pectin solution, and one part water.

*Tobacco pectase* is an esterase whose action is confined to splitting off methyl alcohol from pectin. Neither a protopectinase activity (conversion of water-insoluble pectin into soluble pectin) nor a pectinase activity (breakdown of pectin and pectic acid to reducing sugars and other simple substances) has been observed for the pectic enzyme of tobacco. A secondary effect of the pectase action is the formation of gels of calcium pectates in the presence of calcium ions from pectin solutions. [Wenusch (334) reports that addition of the press juice of tobacco stalks to 10% sugar solutions makes these solutions gelatinize, an effect that is possibly connected with the pectin and pectase content of the tobacco juice.] This enzyme, which is common to many fruits, leaves, and roots, has a pH optimum between 5.5 and 6.5.

In the leaves, the enzyme is fairly resistant to heat treatment. Neuberg and Kobel found that it was not inactivated in tobacco leaves which were first dried in the air and then at 100° C. for 1 minute. According to Kertesz

(114), extracts of the enzyme made from tobacco powder are completely inactivated in 2 min. at 90° C. and in 1.5 min. at 100° C. Davison and Willaman (42) found thermal inactivation of pectase solutions, after a longer treatment, at 68° to 70° C.

Of other esterases, a *lipase* was found in tobacco leaves (1, 179, 183) which may play a role in the conversions of some of the ether-soluble compounds. It is also probable that *chlorophyllase* is present in the leaf tissues.

The importance of the phosphoric acid esters of the carbohydrates for the activation of the latter for alcoholic fermentation and for oxidative degradation has been proved in numerous instances, although it is still doubtful whether phosphorylation is a *conditio sine qua non*, or a minor pathway, for the enzymic conversions of the carbohydrates (185). In the tobacco leaf, *phosphatases* have been identified by Andreadis (1), Baba (3), Neuberg and Kobel (175), Ignatieff (103), Smirnov (260), and Kobel and Scheuer (121). No changes in the activity of this enzyme as a result of curing were detected by Smirnov. The activities were measured by the speed of hydrolysis of the magnesium salt of hexose diphosphate in the presence of leaf extracts.

(c) **Proteases and Peptidases.**—Only a few investigations have been made of the enzymes which hydrolyze tobacco leaf proteins (61, 67, 194, 253, 255, 260). Apparently, these enzymes do not differ from the *proteases* and *peptidases* found in other vegetable materials. The protease and peptidase activity of tobacco leaves is considerably increased (194) during the first or starvation stage of curing of tobacco leaves. At the end of curing, however, a slight decrease (about 10% for the protease and 27% for the peptidase) of the activities was demonstrated (265). From the small amounts of polypeptides which Vickery and Pucher (316) were able to find among the split products of the leaf proteins, they concluded that not only enzymes of the tryptic type but also peptidases share in the enzymic hydrolysis of tobacco proteins. Nito and Kitamura (183) found enzymes of the trypsin and erepsin types in green and cured tobacco leaves.

To the writer's knowledge the question whether *arginase* or *histidase* is represented in the complex of proteolytic enzymes has not been investigated. Smirnov (260) mentions the presence of *asparaginase* in tobacco leaves. It also seems probable that there are enzymes in the leaves which catalyze the various transamination reactions (98) by which amino acids of various types are eventually formed, through a transfer of ammonia from glutamine or asparagine with the formation of the corresponding keto acids.

#### DESMOLASES, DEHYDROGENASES, OXIDASES, AND PEROXIDASES

The description of the chemical effects of curing showed that oxidation processes become increasingly important as curing proceeds. High ten-

peratures, such as those used in flue curing, suppress some of the oxidative conversions, by inactivating the corresponding enzymes. But even in this type of curing the respiratory consumption of carbohydrates and the oxidative changes of protein split products are not completely suppressed.

(a) **Enzymes of the Zymase Complex.**—Neuberg and co-workers tested tobacco extracts for the presence of those enzymes considered as essential components of the complex functioning in the alcoholic and lactic-acid fermentation of carbohydrates.

The enzymes which split glucose (glycolase, aldolase, zymohexase), probably via fructose, into two triose fragments (methylglyoxal, glyceraldehyde, or dihydroxyacetone, all in their phosphorylated forms), together with the enzymes of the various steps of phosphorylation, are often considered as decisive catalysts in the fermentation of carbohydrates in yeast and muscle preparations. They start the chain of reactions which lead to alcohol and carbon dioxide, or to lactic acid, as the final products obtained from the carbohydrates. Neuberg and Kobel (176) and Kobel and Scheuer (121) found *glycolase* in green and dried tobacco leaves. The same authors also demonstrated the presence of an *aldoketomutase* in the leaves. This enzyme, according to Neuberg, catalyzes the oxidoreduction of methylglyoxal to lactic acid, and a related enzyme (*aldehyde mutase*) supports the oxidoreduction (Cannizzaro dismutation) of two acetaldehyde molecules to acetic acid and ethyl alcohol. These findings, considered along with the fact that small amounts of ethyl alcohol and acetaldehyde were detected in tobacco (175), and with the report of Fodor and Cohn (66) that zymase (the enzyme complex associated with the alcoholic fermentation of sugars) is present in tobacco leaves, make it conceivable that there is an alternative path for the oxidation of the carbohydrates to that leading through the citric acid cycle (see page 359). [In this connection, Street's observation that the addition of yeast improved tobacco fermentation is interesting (294).]

This alternative path would have many steps identical with those of alcoholic fermentation. The difficulties in accepting such a scheme are: (1) lack of proof for the oxidation of acetaldehyde in the leaf tissues, and (2) the fact that Kobel and Scheuer were unable to demonstrate the presence in tobacco leaves of decarboxylase (necessary for the decarboxylation of pyruvic acid to acetaldehyde). This negative result is at variance with the findings of Fodor and Reifenberg (67), Kretovich (130), and Smirnov and Petrik (268), who claim to have obtained positive checks for the presence of carboxylase in tobacco leaves. However this may be, the citric acid cycle, for the time being, presents the best founded and most consistent hypothesis of the way in which enzymic oxidation of carbohydrates and related substances is accomplished in tobacco leaves.

(b) **Dehydrogenases.**—So far, the search for oxidizing enzymes in tobacco leaves or in extracts of tobacco leaves has been confined mainly to the demonstration of enzyme complexes which, in their over-all effect, lead to oxygen uptake and carbon dioxide evolution. In addition to these studies on the respiration of leaves or extracts, many authors employ the aerobic oxidation by tobacco extracts of mono- and polyphenols (mostly of hydroquinone or catechol) as a standard for determining the oxidase activity of their samples.

Although these methods of measuring the oxidative power of the leaf enzymes may give valuable information about the relative changes of some oxidation catalysts, one cannot expect from them a complete picture of the enzymic apparatus which promotes the oxidation of many of the leaf components.

It is generally acknowledged that most of the biochemical oxidations are composite reactions consisting of chains of successive single chemical conversions, each of which, as a rule, requires a specific enzyme. Thus, in the citric acid cycle the several mediator compounds are generated from each other by dehydrogenations, isomerizations, decarboxylations, etc., provided the necessary specific enzymes are present for each step. The following substances have been found to be indispensable in biological systems for smooth functioning of the respiratory oxidation of carbohydrates (9, 128, 228, 295):

- (1) *Inorganic substances*: phosphates, magnesium, or manganese ions.
- (2) *Mediator substances*:  $C_4$ -dicarboxylic acids (succinic, fumaric, etc.).
- (3) *Adenine nucleotide* (adenylic acid = adenine + ribose + phosphoric acid).
- (4) *Codehydrogenase* (nicotinamide + ribose + di-[or tri]-phosphoric acid).
- (5) *Cocarcboxylase* (thiamin diphosphate).
- (6) The appropriate proteins to serve as the *apoenzymes* of the coenzymes (4) and (5).

Of these substances, (1) and (2) are known to be present in sufficient quantities in normal tobacco leaves. Furthermore, it is conceivable that parts of the leaf proteins or their digestion products provide (6), and that the nucleotides of the leaf cells contain sufficient amounts of (3). This leaves (4) and (5) as those enzyme system components whose presence in tobacco leaf tissue is to be demonstrated.

In principle, this can be accomplished in two ways. The first is to select as test reactions the transformations which are typical and specific for the enzymes in question. It is known that codehydrogenase catalyzes dehydrogenations of malic, isocitric, and glutamic acids, as well as of glucose, in the presence of the apoenzyme (protein carrier)

and of hydrogen acceptors such as methylene blue. The cocarboxylase acts on pyruvate and on some other keto acids, with the liberation of carbon dioxide. It would be interesting to see whether, and to what extent, these specific substrates are attacked when they are added to tobacco leaf extract, or, better still, when infiltrated into tobacco leaves. The latter method seems more dependable than the one involving extracts, since there is always the possibility that several of the native enzymes either are inactivated during extraction, or are not extractable from the leaves, a contingency especially likely for the apoenzymes.

The indications of a positive carboxylase action of tobacco extracts (see page 370), and the occasional demonstration of dehydrogenating properties of tobacco extracts (68, 149, 194, 260) point to the probability that such experiments, with the proper compounds as substrates, would give further valuable information. Another way would be to attempt the analytical identification of the codehydrogenase and cocarboxylase, or at least of their prosthetic groups, nicotinamide and thiamin. Several good, sensitive methods are available today for the detection and determination of these compounds, so that a direct identification of these coenzymes should not be too difficult. No systematic work has yet been carried out in this direction. It is interesting to note here again the possibility that nicotine may serve as the mother substance of compounds with dehydrogenase properties. [Analytical results have been obtained in this laboratory proving that a large proportion of the nicotine which disappears during fermentation of Pennsylvania Seedleaf tobacco (up to 70% of its initial value, in well-fermented tobacco) is transformed into substances still containing the pyridine ring, but no longer showing the characteristics of alkaloids. This work is being continued.] No investigations as to the presence of thiamin diphosphate in tobacco leaves have been made, to the writer's knowledge.

(c) **Oxidases.**—In order to achieve aerobic oxidation, the enzymes of dehydrogenation require a supplementary system of enzymes by which their hydrogenated forms are again dehydrogenated with the participation of oxygen and the formation of water or of hydrogen peroxide as reaction products. Some discussion as to which of the known enzymes can perform this function has appeared recently (228, 293, 295). It seems likely that *flavoproteins* (yellow enzymes) such as *diaphorase* or *cytochrome reductase* are able to accept the hydrogen from the dihydro form of the codehydrogenase, and bring it into reaction with the oxygen offered in an activated form by *cytochrome c* (iron-hematin-porphyrin). The latter pigment is reoxidized by oxygen of the air, under the influence of cytochrome oxidase (indophenol oxidase). Another possibility is the direct reaction between the hydrogenated codehydrogenase and *o*-quinones (132), with the formation of water or hydrogen peroxide, and of the corresponding diphenols. This latter possibility seems to be particularly interesting for the oxidative changes in tobacco leaves, because oxidation of polyphenols to quinones and tannins in the leaf tissues has been established (see page 336). It seems possible that such processes are coupled with the respiratory oxidation of the carbohydrates of the tobacco leaf, like the mechanism of tea fermenta-

tion postulated by Roberts (225). He has recently suggested this possibility, and also discussed the flue curing of tobacco (224) from this point of view. Nelson and Dawson (174), on the basis of the reactive behavior of the system *tyrosinase* (or phenol oxidase) + tyrosine + ascorbic acid in potato slices and in similar materials, came to the equivalent conclusion, namely, that *o*-quinones produced by the oxidation of phenols or diphenols in the presence of (poly)phenol oxidase (*tyrosinase*) can act in these systems as acceptors of the activated hydrogen of ascorbic acid, carbohydrates, etc., with the formation of water and diphenols. This "shuttle action" continues, according to Nelson and Dawson, as long as activation of the hydrogen of the respiratory compounds is not interrupted, either by exhaustion of these substances or by disturbance of their enzymic activation. As soon as such an interruption occurs, the (poly)phenol oxidase completely converts the phenolic substances which are present to permanent oxidation products (quinones, tannins, melanins). This scheme, already suggested in its basic features by Onslow (193) as a possible mechanism for the respiration in various plant materials, agrees well with some of the observations made with curing tobacco leaves: in flue curing, in which the respiratory oxidation of the carbohydrates is intentionally left incomplete, no appreciable oxidation of phenols occurs, while the opposite is true in air curing, in which a large proportion of the respiratory substances in the leaf tissues is exhausted.

Seen from this point of view, the numerous studies of the oxidase (polyphenol oxidase) (101, 153, 155, 296) of tobacco leaves are important not only for the specific question of oxidation of phenols but also for the entire complex of oxidative changes (10, 12, 17, 22, 24, 25, 68, 110, 140, 174, 179, 183, 191, 194, 223, 260, 267, 297, 301, 326, 349).

The *phenol oxidases* (*tyrosinase*) and *polyphenol oxidases* have recently been described so thoroughly (225, 296) that it seems superfluous to treat them in detail here. Roberts (225) is inclined to identify the oxidase of tea leaves with the cytochrome oxidase. Nelson and Dawson (174) show that the oxidation of phenols, including tyrosine, and of diphenols, as it occurs in the tissues of mushrooms, potatoes, and many other plants, is caused by a copper-containing protein, probably of hemocyanin character. Smirnov and Moroz-Morozenko (267) found the optimum *pH* of tobacco oxidase to be about 6.4. Okuda *et al.* report a *pH* optimum of 7.3 and a temperature optimum of 60° C. (191). Smirnov and Moroz-Morozenko also found that potassium cyanide strongly inhibits its activity, and that the addition of small amounts of copper salts restores its activity almost completely (262). These three observations seem to indicate a close rela-



tionship between tobacco oxidase and the tyrosinase of Nelson and Dawson. But more recent studies of Smirnov and Pshennova (269) cast doubt on this possibility; they found that the tobacco polyphenoloxidase was strongly specific for hydroquinone, and was inhibited by catechol, resorcinol, pyrogallol, and tannic acid, specificities which differ from those of tyrosinase (174). Smirnov's observation of a slight decrease in the activity of tobacco polyphenol oxidase in the presence of large amounts of carbon monoxide (262) possibly indicates a participation of cytochrome oxidase in the over-all effect.

A number of authors have studied the activity of the tobacco oxidase during growth of the plant and treatment of harvested tobacco leaves. Their findings regarding the change of oxidase activity in the course of curing disagree so thoroughly that a detailed report of them seems useless. Suffice it to mention that the effects observed as a result of the curing vary from a complete disappearance of the oxidase action to an increase by as much as 80%. The type of tobacco, age of leaves, kind of weather, type and conditions of curing, and further variables seem to be the cause for these discrepancies. Furthermore, in many cases, the methods of determining oxidase activity are not comparable. High temperatures seem to cause a rapid loss of oxidase activity, an effect in accord with the early disruption of many oxidative processes in flue curing.

Recent investigations by Obabko (188) illustrate the complexity of the effects commonly used as indicators of oxidase activity of tobacco. Obabko's work will be discussed in detail in part II of this study; only three of his observations will be mentioned here, in view of their general bearing on the nature of oxidase activity, and the experimental methods of its determination. He found:

1. Treatment of tobacco at temperatures up to 60–70° C., in a nitrogen atmosphere, does not markedly affect the leaf's capacity to absorb oxygen and evolve carbon dioxide, and the leaf shows all the other exterior and chemical earmarks of regular fermentation.

2. In contrast to the harmlessness of such treatment on the oxidative processes in the tobacco leaves proper, aqueous extracts of the heat-treated leaves show a greatly diminished capacity compared with extracts of untreated tobacco leaves.

According to Svirin (298), oxygen absorption by the tobacco leaf suffers less from heat treatment (80° C.) than does the oxidase activity of the leaf extract. The former decreases by 24–55%, the latter by 80–96%.

3. The oxidative capacity of the aqueous extracts, greatly reduced or destroyed by treatment of the leaves at 60–70° C., can be almost completely restored by heating the leaves, again in a nitrogen atmosphere, at still higher temperatures—up to 125° C. Various facts, however, show that

this restored oxidase activity of the leaf extracts is not caused by enzymes, but probably by the presence of autoxidizable (phenolic?) substances formed in the leaves during the high-temperature treatment, and by catalysts of a nonenzymic nature. The first two of Obabko's findings show clearly how careful one must be in drawing conclusions about enzymic activities and changes of these activities based on experiments with extracts only. The fact that the tobacco leaves retain their oxidative capacity after the heat treatment, *while the extracts do not*, can be explained by either of two possibilities: (1) the existence of two independent oxidative systems in the leaves, or (2) by changes in the extractibility of the oxidases.

Cytochrome oxidase, for example, can be present in the leaves, in addition to phenol oxidase; the former is eventually bound more firmly to the proteins of the leaf tissue. Furthermore, ascorbic acid oxidase has been reported by Okuda, Katai, and Murata (192) as a component of the enzyme system of tobacco leaves; the difference in its pH optimum and temperature optimum from those of the phenol oxidase makes it likely that the two oxidases are different enzymes.

Only further detailed studies can clarify these complex phenomena. Obabko's third observation points to the always existent possibility of pseudo enzymic effects, which actually are caused by nonenzymic heat-resistant catalysts.

(d) **Peroxidases.**—The numerous reports about the peroxidases of tobacco leaves show, when tabulated, a confused picture. In addition to the studies on oxidases, there are a number of special experimental studies (2, 14, 33, 81, 252, 257, 263, 266, 352, 353) aimed at detecting the peroxidases of the leaf tissues, determining their activities, and following the changes in peroxidase activity during growth of the plant and treatment of the harvested leaves. Most of the investigators choose the formation of purpurogallin from pyrogallol and hydrogen peroxide in leaf extracts as the test reaction. Increases and decreases of peroxidase in the leaves, before and after curing, range from +333 to -100%. The pH optimum, according to some of the authors, lies between 6.0 and 6.5, the temperature optimum at about 60° C. To what extent peroxidase supports respiration is unknown. It probably causes the utilization of hydrogen peroxide whenever this compound is produced from the reaction between activated substrate hydrogen and molecular oxygen.

(e) **Catalase.**—The presence of this enzyme in the tobacco leaf, easily detected by decomposition of hydrogen peroxide in aqueous extracts of the leaves, has been studied in very many general, and in a number of special investigations (140, 141, 154, 159, 190, 254, 327, 329).

Whether the catalase, besides its protective function against the excessive accumulation of hydrogen peroxide, is important for the respiratory and other oxidative changes in the leaf tissue, cannot yet be decided. As a rule, it largely disappears during the first stages of curing, unless the development of mold or bacteria introduces a new source of this enzyme. Despite some statements to the contrary, most authors agree on this point. The catalase probably plays only a subordinate role in the chemical changes of the harvested leaf, although its presence may be of considerable importance for the living plant.

(f) **Enzymic Conversion of Nicotine.**—No clear answer has yet been found for the question of whether it is the enzymes, or catalytic agents in general, contained in the tobacco leaf which promote the conversion of nicotine into other compounds, or whether bacterial enzymes are required for this specific reaction. One reason for this uncertainty is the lack of knowledge about the chemical nature of nicotine conversion in the leaf tissues, and the corresponding lack of dependable analytical methods for measuring not only the disappearance of the alkaloid but also the appearance of its transformation products. Discussion in detail of the conversion of alkaloids is reserved for part II of this study, since such conversion is much more intensive during fermentation than during curing.

Some observations seem to indicate that nicotine conversion can be brought about by enzymes or catalysts present in the leaf. Among such observations are: (1) The indications that nicotine is transformed in senescent leaves of the living tobacco plant. (2) The findings of Fodor and Reifenberg (67) that extracts of green, cured, and fermented tobacco leaves, if added to nicotine solutions, cause a partial decomposition of the alkaloid, with the formation of volatile bases (ammonia, methylamine?) which show none of the specific alkaloid reactions. (3) The findings of Larson and Haag (138) on the transformation of nicotine into unknown compounds (excreted in the urine) in the animal body which seem to demonstrate the possibility of an enzymic breakdown of the alkaloid.

On the other hand, Wenusch (338), Weber (330), and Enders and Glawe (60) have carried out experiments which have led them to believe that nicotine conversion can be largely ascribed to the action of certain specifically acting oxidase-, peroxidase-, and catalase-producing bacteria.

In the writer's opinion, neither the purely enzymic experiments with nicotine solution as a substrate, nor the tests carried out with cultures of microorganisms, seem to be at a stage at which definite conclusions can be drawn as to the nature of this important chemical process in the tobacco leaf.

Thus, our present knowledge of the really decisive enzymic factors in tobacco curing is scantier than our knowledge of the chemical changes which this process causes in the leaf tissues. Obabko's observations show that many of the older studies, which were made with tobacco extracts, may need revision. However, one may reasonably assume further progress in this special field of plant biochemistry, if investigations are carried out as carefully and thoroughly as those in the study of animal tissues.

Aside from practical considerations, the mysteries of the metabolism and catabolism of the tobacco leaf present a challenge for basic scientific research.

The second important phase of tobacco processing, commonly called fermentation, is not, as is sometimes assumed, a mere continuation and completion of the chemical changes begun in the curing process. On the contrary, new types of conversions make their appearance which seem as important for the well-controlled manufacture of smoking tobacco as the changes caused by curing.

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# THE ACTIONS OF THE AMYLASES

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## I. Introduction

(The amylases are hydrolytic enzymes which, as their name clearly indicates, promote the decomposition of starch (*amylum*). Actually they attack glycogen and certain dextrans as well.) It is essential at the outset to distinguish between enzymes which promote the breakdown of the above-named substrates by hydrolysis, by the use of water only, and the phosphorylases, which act on starch and glycogen with the intervention of phosphate. The phosphorylases both synthesize and break down, and in the latter case the final product is glucose-1-phosphate. This action is not as yet of any industrial significance and for this reason, apart from questions of definition, it will not be dealt with in this review.

(Amylase action is of great industrial and biological importance, but is not, even now, completely understood after more than a century of research. The reasons are rather obvious. Starch, the usual substrate, presents unusual difficulties, including its biological nature with granules of different sizes, shapes, and microscopic characters, its composite chemical nature, the problems encountered in separating it into its components, and, above



all, its large and complex molecules whose structures could not be elucidated without highly specialized techniques. Acid hydrolysis imposed drastic conditions and gave little information as to the structure of starch, whereas the gentler enzyme action involved the use of what was usually a mixture of enzymes—a mixed substrate acted upon by a mixture of enzymes. It is small wonder that contradictory conclusions have appeared and that progress has been slow.

After controversies lasting for nearly forty years, the hypothesis originally put forward by Maquenne, that native starches consist mainly of two chemically different substances, amylopectin and amylose, may now be said to be established as substantially correct.) Of the various devices that have been used to bring about the separation, one of the most satisfying in many ways was that of extraction of the amylose from the granules without even what may be described as the violence of boiling. Tanret (78), Baldwin (3), and more recently Meyer (53) have developed this method, in which care is taken to avoid the complete disruption of the granules by heat. Whatever processes are employed, they should be as gentle as possible. It is claimed that even prolonged grinding of the dried, natural starch (corn, wheat) in a ball mill disrupts the molecules (5, 46). In practice, Schoch's treatment of autoclaved starch with butanol (72) is effective.

Electrophoresis, originally applied by Samec (70) to autoclaved starch paste, has also yielded useful results with potato starch, the "amyloamylose" of Samec possessing much the same properties as amylose. Even if autoclaving is avoided, this method seems useful for the preparation of small yields of amylose from potato (36) and corn starches (12). The separation has not been so successful with wheat starch (76).

(It seems that most natural starches contain 20–25% of amylose, the bulk of the remainder being amylopectin. The former consists of long unbranched molecules of roughly 300 glucose units average length (54), the latter of branched or laminated molecules, the average length of a terminal branch being from 12 to 18 units. Amylose is responsible for the blue iodine coloration, amylopectin for a red coloration which is masked to ordinary vision by the blue, *i. e.*, without spectrophotometric analysis, even if only a relatively small quantity of amylose is present. These two are the components with which we are essentially concerned in considering amylase action.) We are not concerned with other carbohydrate constituents, such as "amylohemiacellulose" in cereal starches, and the noncarbohydrate constituents, although phosphate has contributed greatly to the confusion and may yet be proved accountable for the resistance of certain limit dextrans to amylolytic degradation.

## II. General Features of $\alpha$ - and $\beta$ -Amylases

(The outstanding features of amylase action are the liquefaction, dextrinization, and saccharification of starch,) to which may be added the erosion and disintegration of raw starch granules which must take place in the plant. Until definite and confirmed evidence is obtained of an enzyme with a separate starch-liquefying function, there is no need to postulate more amylolytic enzymes than the  $\alpha$ - and  $\beta$ -types to account for all the known phenomena, although their activities are certainly supplemented by that of maltase, and, apparently, by those of other glucosidases.

(Briefly, the  $\alpha$ -amylases break the starch molecules by hydrolysis of  $\alpha$ -glucosidic links into dextrins of relatively low molecular weight, thereby "liquefying" the starch) destroying its capacity to form colors with iodine, and increasing its susceptibility to the  $\beta$ -amylase. Later, but overlapping this process, maltose and glucose are slowly formed until about 80% of the starch is in the form of these sugars, mostly maltose, and the remainder in the form of "limit dextrins."

(Beta-amylase (there is only one found alike in sweet potato (20) and ungerminated grains of barley and wheat, soybean, etc.) saccharifies the starch,  $\beta$ -maltose being formed) leaving as residue a succession of amyloid dextrins which retain much of the starchy character of the original substrate. Dextrinization in the true sense does not take place and liquefaction is correspondingly slow. When 53-60% of the theoretical yield of maltose has been formed, the action ceases, no glucose being formed. The residual " $\beta$ -limit dextrin," as originally isolated after the action of slightly impure  $\beta$ -amylase such as that of ungerminated barley, has been best known as erythrodextrin (80) or  $\alpha$ -amylodextrin (1). It should be made clear that, admixed with the  $\alpha$ -enzyme, the  $\beta$ -amylase contributes to the liquefying and dextrinizing activities, so that, in the measurement of the  $\alpha$ -amylase in the presence of the  $\beta$ -form, allowance must be made for the latter.

In reviewing what is known of the actions of these two amylases, it is convenient to deal with the  $\beta$ -type first, as it is the better and more easily understood.

### 1. $\beta$ -Amylase

The first question to be answered is: what exactly is  $\beta$ -amylase? Is it the enzyme which promotes hydrolysis of most starches to the stage of 60% of the theoretical maltose, as is usually taken to be the case? Or is it the same enzyme which, after pretreatment at pH 3.4, and under suitable conditions of action, cannot hydrolyze beyond 53% as demonstrated

by the results of Blom and co-workers (6)? These results have been fully confirmed in unpublished work by the reviewer and the conclusion can be drawn that the true  $\beta$ -amylase completes its action at 53% of the theoretical maltose. The difference between this limit and the various limits from 60 to 67% (1, 23) are attributed to a separate enzyme which does not function at pH 3.4, and might well be a trace of  $\alpha$ -amylase. Indeed, the addition of suitable small traces of the latter to what we will, for convenience, term the " $\beta$ -53"-amylase causes it to behave as the " $\beta$ -60" enzyme. One thing is certain, namely, that the  $\beta$ -53-amylase acting on, for example, Lintner soluble starch at pH 3.4–3.6, ceases action at the 53% stage, while there remains active enzyme in the reaction mixture, as can be easily demonstrated. The only inference to be drawn is that the enzyme which functions at pH 3.4 to promote fission of maltose to the 53% stage is not able to continue the procedure at pH 3.4. Whether the hydrolysis equivalent to the difference between 53 and 60%, and which is promoted at pH 4.6, is due to a trace of  $\alpha$ -amylase or to a different enzyme, or is associated with certain retrogradation effects cannot be discussed here. If the substrate were strongly ionized (as in the case of protein, for instance) one could accept the hypothesis that the active  $\beta$ -enzyme could hydrolyze certain links at pH 4.6, but not at pH 3.4. The description that follows can apply alike to the  $\beta$ -53 and the  $\beta$ -60 enzymes, except as regards the limit of action. The importance in the decision consists in its effect on the calculation of the mean chain length of branches in the amylopectin molecule.

The specific action of  $\beta$ -amylase is as follows:

(a) Only  $\alpha$ -1,4-glucosidic linkages are attacked. The evidence for this rests on the work of Haworth and Percival (28), which indicates that the prevailing linkage in the starch molecule is the 1,4-glucosidic linkage, and the application of the Freudenberg rule of optical superposition, from which the conclusion is drawn that these links are almost wholly  $\alpha$ -glucosidic linkages as in maltose (23). The  $\beta$ -amylase must break these, since there cannot be other linkages to break in the case of amylose [see (c) below], and certainly not enough in the case of starch to account for a yield of 53% of theoretical maltose.

(b) Fission commences with the second  $\alpha$ -1,4-glucosidic bond from the nonreducing end of a chain (or branch). When this has been broken and a maltose molecule formed and removed, the second link in the remainder of the chain is in turn attacked. Except that he did not specify the non-reducing end, this hypothesis was first advanced by Ohlsson (67). It was based on the fact that osmotic pressure measurements during the hydrolysis indicated no increase in the number of nondialyzable particles present.

Second, it had been established that only maltose and no glucose is split off from the amyloid molecule (1). Third, it has since been shown that analysis of the products of the enzyme action at any stage, particularly the early stages of the hydrolysis, reveals the presence of only maltose and a residual starchy dextrin (17). Fourth, the uniformity of the velocity of saccharification even in dilute solutions of starch (52), a zero molecular reaction until about 40% maltose has been liberated, can best be explained by this mode of action which maintains a constant molar concentration of substrate although the concentration per cent is continually falling. Fifth, as to the end of the molecular chain attacked, Brown and Millar (10) oxidized "maltodextrin" with mercuric oxide to the corresponding "dextrinic acid" and found it to be hydrolyzed by malt amylase ( $\alpha + \beta$ ) yielding maltose and a dextrinic acid of lower complexity. More recently, this has been confirmed using  $\beta$ -amylase and, later,  $\alpha$ -amylase (62). Starch and dextrans were oxidized by hypiodite. Notwithstanding the fact that no account appears to have been taken of the inactivation of amylases by iodides (31) in some of these investigations, they indicate that the reducing end of the molecule is not involved and that the attack must therefore commence at the nonreducing end or ends.

Thus the specificity of  $\beta$ -amylase and its mode of attack are somewhat analogous to those of certain peptidases which in effect could attack suitable protein "chains" from one end only and progress along them.

(c) If no branching occurs in the molecular chain, hydrolysis continues to completion, maltose being the sole product unless the presence of an odd number of glucose residues results in the last three of them, constituting maltotriose, remaining unattacked. Amylose, according to the evidence of Meyer (54), which, however, has been criticized (26), consists of unbranched chains. It is completely hydrolyzed to maltose (52). The absence, for example, of 1,6-linkages in amylose seems fairly certain although the proof is scarcely satisfactory. Other forms of amylose, such as the synthetic product of Hanes (24, 26) and the amyloamylose prepared by electrophoresis, have also been hydrolyzed to completion (18, 71) or very far beyond 53 or 60%, even when care has been taken to maintain mild conditions of preparation (36) such as would reduce to a minimum the risk of disrupting a branched molecule. A significant point is that the  $\beta$ -53- and  $\beta$ -60-amylases from barley hydrolyze amylose much more slowly than soluble starch under identical conditions. This strongly supports the view that the molecule of amylopectin presents many nonreducing chain ends to the enzyme, whereas the molecule of amylose presents only one (or relatively very few). Incidentally, this slowness introduces difficulties in that

a certain amount of reversion of the amylose to insoluble and insusceptible forms takes place during the enzyme action which, as a result, may not be completed. A corresponding change takes place in the solutions of amylose on storage; thus, the enzyme action must be performed on newly prepared solutions of amylose (36, 52, 64, 76), and a fast reaction promoted.

The interpretation of the results of action of  $\beta$ -amylase on amylose fractions is complicated, not only by these tendencies to reversion even during the hydrolysis which must of necessity be prolonged, but also by the possible presence of amylopectin due to imperfect separation of the starch components.

It is not surprising that the blue iodine reaction is given by amylose throughout its hydrolysis by  $\beta$ -amylase until 90% or so of theoretical maltose has been formed (71). When starch itself is the substrate, the residual dextrin gives a blue coloration—often purple when the reaction has proceeded to 60%. A trace of undegraded amylose gives a blue reaction, which will mask the red color due to amylopectin and its degradation product,  $\alpha$ -amylodextrin, unless spectrophotometric methods of observation are employed.

(d) Branched molecules, *i. e.*, chains of glucopyranose residues linked by  $\alpha$ -1,4-glucosidic bonds, but also branched or laminated, whether by  $\alpha$ -1,6-glucosidic linkages or otherwise, are not completely hydrolyzed. The process commences, as with amylose, at the nonreducing ends but stops at the near approach of a branching. Clearly, when either of the glucopyranose residues of the next pair to be removed as maltose has a hydroxyl group other than 1 and 4 engaged, the highly specific  $\beta$ -amylase can no longer function. Actually, this arrestment must occur one glucose residue short of the one involved in the branching of each terminal branch (27), and with pure preparations of  $\beta$ -amylase, a final cessation of action occurs. The product is best termed " $\beta$ -limit dextrin" as by Myrbäck, or perhaps even better, " $\beta$ -53 dextrin" or " $\beta$ -60 dextrin" as by Blom (6). The usual erythrogranulose or  $\alpha$ -amylodextrin corresponds to Blom's " $\beta$ -60 dextrin" and is more soluble and slightly less starchy in character than the  $\beta$ -53 dextrin.

(e) The maltose formed is in the  $\beta$ -form (45, 67) whether the substrate is amylose, amylopectin, or starch (19).

Assuming the structures of amylose and amylopectin to be as postulated above, the specificity of action described so far accounts for almost all the known facts: the slow liquefaction, the delayed changes in iodine coloration, the appearance of maltose as sole sugar, the relative rates of hydrolysis of amylose and starch, the zero molecular character of the first 40%

hydrolysis of starch (on which Kjeldahl's law is based), the complete hydrolysis of amylose, partial hydrolysis of amylopectin, starch, and glycogen, and the absence of action on  $\beta$ -limit dextrin. A slight action on the latter, and indeed the recorded kinetics of action on  $\alpha$ -amylodextrin (1, 22, 23), can be accounted for by the assumption that the  $\beta$ -60-amylase contains an additional enzyme as already discussed, and that crude barley amylase is almost invariably contaminated with a little  $\alpha$ -amylase (15, 29, 56). The resumed action on  $\alpha$ -amylodextrin which has been previously attacked by  $\alpha$ -amylase, reported by many authors (18, 22, 27, 40), is also consistent with the specificity described.

The amount of  $\alpha$ -amylase present in a crude  $\beta$ -amylase preparation varies with cultivation and varietal factors of the grain from which it is prepared (15, 29) and, in general, appears to be low with wheat (77) and barley, and even less with soybean flour (65). It depends also on the method of extraction and care in the use of antiseptics, since  $\alpha$ -amylase secreting bacteria may be present on grain (as demonstrated in unpublished work in the reviewer's laboratories), the method of separating the enzyme preparation from the crude extract, and above all on pH and temperature maintained during preparation. The  $\alpha$ -amylase in grain is more susceptible than the  $\beta$ -amylase to ethyl alcohol and acidity but is more stable at high temperatures. The limits of  $\beta$ -amylase action on starch reported in the literature vary, as already indicated, partly because the enzyme employed has varied in purity from one appreciably contaminated with  $\alpha$ -amylase to " $\beta$  53" preparations acting at pH 3.6. A certain tendency to "drift" when the saccharification limit has been attained is noticeable and care is needed in forming an estimate of what change is attributable to experimental error. Careful controls, maintained in parallel with the prolonged hydrolysis, must be made. When all these precautions are taken, it still appears that the limit of hydrolysis is greatly dependent on the method of preparation of the starch substrate (22, 48), particularly preheating treatment (36, 79) and treatment with acid, as with Lintner starch. It is not known with certainty whether the limits of hydrolysis of starches of different origins (excluding the "waxy" starches) vary at all; they certainly do not vary much. This seems to indicate that the proportions of amylose and amylopectin in such starches do not vary widely. Certain minor differences between the  $\beta$ -limit dextrins derived from the cereal and root starches have been recorded (49). In any case, these limit dextrin preparations contain fractions precipitable by alcohol of widely different mean molecular weights as indicated by diffusion methods (56).

The theory of the action of  $\beta$ -amylase has one or two imperfections. The

facts that only *alternate*  $\alpha$ -1,4-glucosidic links are split and that maltose is liberated in the  $\beta$ -form have been explained (23), but not satisfactorily enough. The two facts may be in some way connected and a more perfect or complete explanation may one day be forthcoming which will embrace them both.

## 2. $\alpha$ -Amylase

The  $\alpha$ -amylases from different sources, the pancreas, saliva, urine, blood, bacteria, molds, malt [dextrinogenamylase (67)], differ among themselves in respect to such properties as stability, pH optima, and so on, but not to their specific actions on starch. As regards any one of these  $\alpha$ -amylases, the fact may now be accepted that it is a single enzyme which is responsible for erosion of starch granules, liquefaction of paste, dextrinization, and the slow subsequent production of maltose and glucose, leaving limit dextrans which are of comparatively very low molecular weight. The further breakdown of these limit dextrans may well be the work of another enzyme or enzymes. Further, it is the general opinion of workers on the amylases that although  $\alpha$ -amylases from different sources exhibit certain differences in kinetics and limits of action, these are mainly to be accounted for by varying affinities for their various substrates, starch, dextrans, etc. Even Ohlsson's dextrinogen amylase, usually termed  $\alpha$ -malt amylase, cannot be segregated from the others although it exhibits a certain individuality in some respects, particularly glucose formation (61). Judged by its specificity, there is one  $\alpha$ -amylase with a range of affinities for its substrates, but the range for any one substrate is narrow and not comparable with the corresponding phenomena among the  $\beta$ -glycosidases (69).

It has been difficult to establish the fact that only one enzyme is involved, but many attempts to fractionate  $\alpha$ -amylases have failed. This matter is again discussed under the heading "criteria of purity."

The specific action of  $\alpha$ -amylases to be described is essentially the same whether amylose or amylopectin is the substrate. In the former case, the action ultimately goes to completion.

The action of  $\alpha$ -amylases may be described as follows:

(a) Dextrinization and, in consequence, liquefaction or destruction of viscosity is the primary function. The amylose and amylopectin molecules are broken down by fission of  $\alpha$ -1,4-glucosidic linkages into  $\alpha$ -dextrans of small molecular weight.

The conception of a dextrinizing enzyme, as distinct from a saccharifying one, was suggested even before Wijsman's demonstration (80) of them in malt diastase by differential diffusion on gelatin starch plates. It was

Ohlsson (67) who first demonstrated the essentially dextrinizing character of the enzyme by measurement of osmotic pressure, which increased to almost ten times as much during the early stages of malt  $\alpha$ -amylase action. Later determinations by physical methods have indicated that these dextrins have molecular weights varying from 1000 to 3000 (57). The presence of dextrins rather than maltose had, in the meantime, been demonstrated by alcohol fractionation methods (17), and Kuhn's (45) mutarotation observations which had been attributed to  $\alpha$ -maltose had been confirmed and accounted for by the formation of  $\alpha$ -dextrins (19). During this early stage of the action, some types, such as malt  $\alpha$ -amylase and salivary amylases, precipitate with an ingredient of the substrate, amounting to 3 to 5% in the case of starch, or otherwise cause it to flocculate (22, 34).

After the rapid fall in viscosity, the most pronounced feature of the amylase action is the change in the iodine reaction color from blue through purple to red-brown, followed by a slower transition through orange to colorless. The quantity of iodine added is a matter of some importance and evidently it is divided among some of the dextrins. Examined by a spectrophotometric method, it is seen that the first stages of digestion are accompanied by decreasing extinction in the longer wave lengths, *i. e.*, increased transmission of the red end. This process continues, accompanied later by a fall in extinction values throughout the spectrum (25). The close general approximation of the curves—extinction value plotted against mean filter transmission (or wave lengths)—in the case of salivary, pancreatic, malt  $\alpha$ -, and *Aspergillus* amylases, is part of the evidence in favor of their fundamental identity. It is true that the next stage of  $\alpha$ -amylolytic action, the release of maltose and glucose, may make greater progress with some amylases than others before the process of dextrinization is complete, with the result that the percentage of apparent maltose formed, or  $R_m$  value, at the stages of various iodine reactions, differs among the amylases. Thus at the achromic point, or final disappearance of iodine coloration as nearly as it can be judged, the percentages of apparent maltose ( $R_m$ ) are (25): malt  $\alpha$ -, 30; *Aspergillus*, 44; salivary, 46; pancreatic, 55. This value in the case of malt  $\alpha$ -amylase is independent of starch concentration, but increases with the latter in the case of *Aspergillus*, pancreatic, and salivary amylases (25). Apparently malt  $\alpha$ -amylase has a greater affinity for the  $\alpha$ -dextrins (considered as a mixed whole) than the other  $\alpha$ -amylases mentioned. In consequence, the rates of saccharification of dextrins promoted by the latter enzymes are more dependent on dextrin, and therefore, original starch concentration, than is the case with malt  $\alpha$ -amylase. At low concentrations the dextrins cannot compete successfully



for the enzyme with the unchanged starch or large molecular dextrans. As a consequence, dextrinization is complete before much saccharification can occur.

The evidence that  $\alpha$ -1,4-glucosidic linkages are broken is the same as that in respect to  $\beta$ -amylase action (23, 28). Amylose, which apparently contains such linkages exclusively, is completely hydrolyzed by  $\alpha$ -amylase (64, 71). That  $\alpha$ -1,6-glucosidic linkages are also broken at this stage, can only be inferred from what is known of the immediate action of salivary amylase on  $\beta$ -limit dextrin ( $\alpha$ -amylodextrin) (27). Even in a few seconds, this dextrin is changed so that it becomes susceptible to the renewed action of  $\beta$ -amylase. This could be explained by the fission of  $\alpha$ -1,4-glucosidic linkages between the branching points. But a characteristic feature of this action is destruction of viscosity, and viscosity is a feature of amylopectin, as are, also, side branchings. It cannot yet be decided whether the fission of  $\alpha$ -1,6-glucosidic links in amylopectin and  $\beta$ -limit dextrin actually occurs, and even if it does, as is probable, whether it is promoted by the  $\alpha$ -amylase itself or by a component enzyme which has not yet been successfully separated from it.

It is not yet known whether the  $\alpha$ -1,4-glucosidic links are attacked in a random or planned manner, although much speculation on this point has appeared in the literature in recent years (50, 61). Experimental evidence indicating that certain  $\alpha$ -dextrans are completely hydrolyzed by  $\beta$ -amylase and therefore contain none other than  $\alpha$ -1,4-glucosidic links, while others *per contra* must contain anomalous links, has been used by Myrbäck (61) in arguments in support of a planned attack upon the amylopectin molecule by  $\alpha$ -amylases.

(The marked slowing down in the rate of reaction at about 33% apparent maltose—dealt with later—exhibited particularly by  $\alpha$ -malt and bacterial amylases, led to the most plausible idea that the essential function of such amylases was to split starch into dextrans containing six glucose residues (23). Experimental evidence derived mainly by fractionation of the hydrolytic products at this and other stages of the reaction has shown, however, that a mixture of dextrans of widely varying character as denoted by solubility in alcohol, reducing power,  $[\alpha]_D$ , and so on, is in fact formed (34). If, however, malt diastase is allowed to act on starch paste at 74° C., which is virtually the same as malt  $\alpha$ -amylase action, until the resting stage is attained, there is an appreciable quantity of a dextrin—called maltodextrin by Baker and Hulton—which corresponds to the expected one, i. e., containing six glucose residues (2). At the stage  $R_m = 33$ , about 23 units of the  $R_m$  value are to be ascribed to the mixture of dextrans (9, 34, 57, 75)

and the remaining 10 to maltose and glucose. Since about 90 parts of dextrin possess reducing power equivalent to 23 parts of maltose, the *average* molar weight of the mixed dextrans must be equivalent to about 8 glucose units. Allowing for the maltose which has already been split from them by the overlapping of the dextrinization and saccharification processes, these dextrans would have an average molar weight of about 1500 at the time of formation, which agrees closely with the determined figure, 1000–3000 (57) by physical measurements and the yields and reducing powers of the isolated dextrin fractions (34). Evidently only a small fraction, about one-tenth, of the  $\alpha$ -1,4-glucosidic links in the starch substrate molecules is broken in the process of dextrinization. Almost the whole of the liquefaction of starch paste is completed with an exceedingly small liberation of reducing groups.

(b) Saccharification follows dextrinization, overlapping it. This involves the fission of  $\alpha$ -1,4-linkages which had escaped previously. While  $\alpha$ -maltose is the principal product, it is not surprising that glucose, too, is formed at this stage concurrently with maltose. Some experimental evidence suggests that the appearance of maltose precedes that of glucose. Thus, Somogyi traced the appearance, in the reaction mixture undergoing hydrolysis by  $\alpha$ -amylases from animal sources (urine, blood, saliva), of dextrans which appeared first, maltose, and finally, glucose, the last to appear (75). Figure 1 shows the type of curves obtained in such experiments. The determination of maltose and glucose depended on the use of a bakers' yeast which in alkaline solution—about pH 8.4 in the external medium—ferments glucose rapidly and immediately, whereas the commencement of fermentation of maltose is delayed by an induction period until after that of glucose is finished. While it would be more satisfying if the glucose had actually been isolated from the mixture and its presence established by chemical means, it may be concluded that Somogyi's experiments prove that glucose makes its appearance much earlier in the hydrolysis than had been previously believed. He concluded that the glucose was produced from certain of the dextrans. This had been repeatedly indicated by the results of previous workers, ever since Baker (1) had found it among the products of action of barley diastase (which always contains a little  $\alpha$ -amylase) on  $\alpha$ -amylodextrin, and Ling and Davis (47) in those of heated malt diastase ( $\alpha$ -amylase) on starch. The collected evidence of the years up to 1938 indicated that the appearance of glucose was associated with the action of the  $\alpha$ -amylase, not the  $\beta$ -, and with far-driven hydrolysis, and in particular was ascribed to the breakdown of the amylopectin part of starch (22). Since the latter is derived from the amylopectin, it may still be main-

tained that the glucose is derived from this portion or component of the starch.

More recently, Somogyi's results have been confirmed by Myrbäck (58), who used malt  $\alpha$ -amylase and the same fermentation device. He concluded that glucose was formed from the outset of the enzyme action and directly from starch, and that malt  $\alpha$ -amylase differed from the other  $\alpha$ -amylases in this respect (64). Amylose, which by separate experiment was found to be hydrolyzed to completion by  $\beta$ -amylase, was broken down to maltose and a small quantity of glucose by malt  $\alpha$ -amylase (64) (Ohlsson's dextrinogen amylase). In view of the approximations imposed by the fermentation method and the fact that Meyer's amylose as employed by Myrbäck is not unadmixed with amylopectin, it seems unsafe to conclude that any glucose is formed from amylose. If any trace of glucose is formed, it can only be due

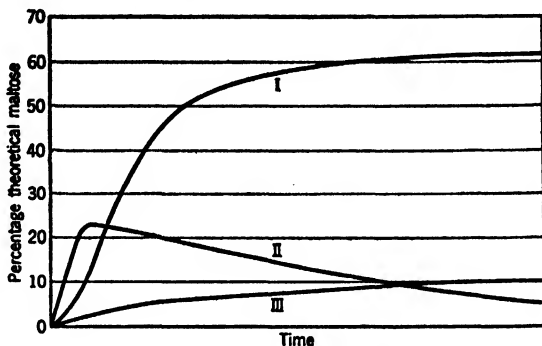


Fig. 1.—Reducing power of dextrins, maltose, and glucose. Curve I, maltose; II, dextrins; III, glucose.

to either (1) branchings in the amylose molecule or (2) the complete breakdown of chain molecules containing odd numbers of glucose residues, thus leaving the residual glucose. It is extremely improbable that all amylose molecules are of the same chain length, and if there is a considerable variation among them in this respect, then some, presumably half, will be built up of an odd number of glucose residues. On the whole, one would expect only a negligible trace of glucose, about 0.04% of the starch to be derived in this way from amylose. The glucose is liberated almost entirely, if not solely, from the products of breakdown of amylopectin, and it would throw light on the mechanism of this breakdown to know just when the glucose makes its first appearance (27). From the results of Somogyi (75) and Myrbäck (58, 64) who used starches as substrates, it seems that dextrins are the first reducing products to appear, maltose making an unmistakable

appearance when the reducing value is about  $R_m$  14–21, while Blom *et al.* (9) furnished results indicating an earlier appearance in the case of the bacterial  $\alpha$ -amylase, "superclastase." As regards glucose, the fermentation method gives no unmistakable evidence of the presence of glucose until  $R_m$  has reached a value such as 28. Indeed, Blom's (9) results do not indicate its presence even then. More satisfactory evidence, with its experimental error capable of more accurate assessment, has been obtained by fractionating the hydrolytic products at certain stages of the amylase action. Such procedure has indicated glucose as being present in the hydrolytic products of potato starch by  $\alpha$ -malt amylase at the stage  $R_m$  33, but only a trace in the case of a bacterial amylase at  $R_m$  40 (34). With whatever fission the formation of glucose is associated, *e. g.*, fission of the amylopectin molecule at or near the branchings, this fission apparently does not occur until dextrinization is over, and Somogyi's conclusion that glucose is split from certain dextrans appears to be substantiated.

The rate at which glucose makes its appearance varies with  $\alpha$ -amylases from different sources (58), depending no doubt, as will be shown, on the relative affinities of these amylases for starch components and their dextrin fission products. Assuming the specific identity of these  $\alpha$ -amylases, the very fact that such variation occurs in the stage and rate of glucose formation, is a confirmation of the view that glucose is split off from dextrans and not from starch directly. Malt  $\alpha$ -amylase appears to produce more glucose than some of the other  $\alpha$ -amylases (58). This, too, may be a question of affinity for certain dextrans which yield glucose on fission, or, on the other hand, it may be due to the presence in malt  $\alpha$ -amylase preparations of a separate enzyme, presumably an  $\alpha$ -glucosidase. The action of preparations of  $\alpha$ -glucosidase from fungal sources on limit dextrans, has been reported (37, 38). Meyer (51) has claimed that an  $\alpha$ -glucosidase obtained from yeast promotes hydrolysis of  $\beta$ -limit dextrin, liberating glucose. He concluded the  $\alpha$ -1,6-glucosidic linkages were broken. However, the enzyme described seems to be identical with a preparation of yeast maltase. The reviewer has not yet succeeded in preparing yeast maltase (employing the method used by Meyer) free of amylase derived from the yeast, as indicated by its action on starch. Under the conditions of Meyer's experiment, such amylase could account for the liberation of maltose from the dextrin, and the maltase for its fission to glucose. The experimental evidence does not warrant the conclusion that glucose was split from the dextrin by an  $\alpha$ -glucosidase. On the other hand, it has been found that  $\alpha$ -amylases have no action on  $\alpha$ -glucosides (8, 69). There is need for further search for evidence of the action of an  $\alpha$ -glucosidase on this dextrin.

(c) It is well known that liquefaction and change of iodine coloration are the most marked manifestations of the action of the  $\alpha$ -amylases. But even when measured by increase of reducing matter, the course of the reaction with most  $\alpha$ -amylases is sharply divided into two stages, a relatively rapid one to a point somewhere between 30–50% theoretical maltose, followed by a much slower one becoming ever slower until it ceases at 80–90%. The sharp bends in the curves (see Fig. 2) are too pronounced for

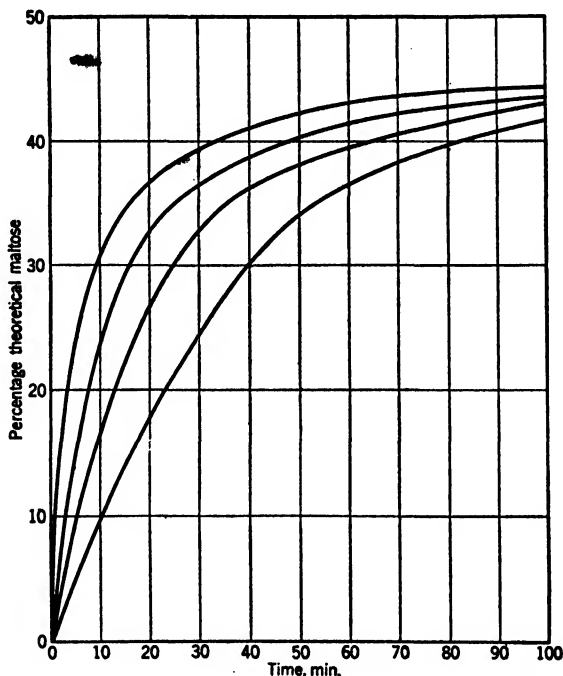


Fig. 2.—Actions of varying quantities of  $\alpha$ -amylase. The final limits after several days (about 80%) are not shown.

any other explanation than that a slow reaction follows a faster one with some overlap. The effect is more pronounced with dilute solutions of substrate. The latter is also the case, apparently, if relatively high temperatures are employed and is partly due to enzyme inactivation.

The evidence available leaves no doubt that the same enzyme is responsible for both the rapid and slow stages of the reaction, although the existence of two enzymes would be a simpler hypothesis. However, attempts

to fractionate these hypothetical enzymes have failed. The alternative explanation of one enzyme possessing widely different affinities for the respective substrates, which compete for the enzyme, may now be accepted with confidence. Apparently the affinity of the enzyme for starch is so much greater than it is for the mixed dextrins present when  $R_m = 35-40$  that this accounts for the phenomena. Unpublished results obtained by the reviewer before war conditions brought such work to a close clearly indicate this in the case of malt  $\alpha$ -amylase. The affinity constant,  $K_i = 0.024(\%)$  (16), was obtained for malt  $\alpha$ -amylase and Lintner soluble starch, whereas the corresponding value for the mixed dextrins present at  $R_m 35-40$  produced from the same specimens of starch, was about 2. The slow stage of the reaction consists in the fission of dextrins for which the enzyme possesses ever smaller and smaller affinity. Moreover, the reaction also slows down because it is, by this time, of the apparent monomolecular type, the velocity being limited by substrate concentration. The variations in the values of  $R_m$  at the point of inflection when starch concentration, enzyme concentration, etc., are varied, are easily understood in the light of these affinity constants, as are also the variations according to the source of the  $\alpha$ -amylase employed. There are good reasons to suppose that  $\alpha$ -amylases of various origins possess different affinities for one and the same starch, beside varying in affinity for the dextrins into which amylopectin and amylose may be split. The adsorption of  $\alpha$ -malt amylase by rice starch granules (7, 32) is presumably due to the high affinity of this amylase for starch.

(d) *The limit of hydrolysis.*  $R_m$  value after prolonged action is about as high in the case of malt  $\alpha$ -amylase as with  $\alpha$ - and  $\beta$ -amylases employed together, although possibly more glucose is formed when  $\alpha$ -amylase acts alone. Results of the type shown in Figure 2 are obtained (35) by using hypoiodite titration, enough enzyme to promote very rapid hydrolysis, and a starch concentration of not more than 2%. If varying doses of the enzyme all lead to the same limiting value, within the experimental error, and it is found that much of the original amylolytic activity is retained, it may be assumed that the limit is a true one. Whenever these conditions are fulfilled the limit of action is found to be at  $R_m 80-90\%$  (not shown in Figure 2), but this limit differs among the  $\alpha$ -amylases. This may be, at least in part, due to varying quantities of glucose among the final fission products. But if the residual dextrins are recovered and the yield of them determined by suitable means, certain differences are found. Malt  $\alpha$ -amylase and taka-amylase leave about 20% of limit dextrins, whereas pancreatic and salivary amylases leave about 25 and 27%, respectively (59).

If these limit dextrins are freed from sugars and redispersed, it is sometimes found that they can be attacked afresh by the enzymes that produced them. This was shown for liver amylase (31) and has been more recently reported in the cases of pancreatic and malt  $\alpha$ -amylases (60). There are grounds for believing that the enzyme has such low affinity for the limit dextrins that the other fission products, maltose and glucose, can, by their inhibiting effect (31, 45, 48, 81), bring the reaction to a standstill. Alternatively, the amylase preparation which functions to split the limit dextrin at the second attempt, does so by virtue of an additional enzyme, *e. g.*, a glucosidase present in the preparation which loses its activity during the long first reaction, and so fails to split the limit dextrin the first time. These limit dextrins range from three to six glucose residues per molecule, but more evidence is needed than is at present available to the reviewer to show what these dextrins are, and what glucosidic linkages occur in them. However, one important observation should be mentioned. Among these products, in the case of malt  $\alpha$ -amylase and maize starch, a trisaccharide has been isolated (63) which contains three glucose residues, two of which are linked together as in maltose, and two as in Fischer's isomaltose (by  $\alpha$ -1,6-glucosidic links). The evidence on which the above conclusion is based includes a reducing power equivalent to a molar weight of 480, molar weight by velocity of diffusion 495, and  $[\alpha]_D +124^\circ$ . From Freudenberg's rule of optical superposition, the value of  $+153^\circ$  for  $[\alpha]_D$  may be calculated for maltotriose and of  $+125^\circ$  for a "triglucide" containing two  $\alpha$ -1,6-glucosidic (isomaltose) bonds. Investigation of the above trisaccharide by methylation methods also indicated the constitution outlined above (63). Calculation also shows that a polysaccharide built up of glucose residues linked by  $\alpha$ -1,6-glucosidic links would have  $[\alpha]_D +180^\circ$  (68), whereas amylose containing only  $\alpha$ -1,4-glucosidic links would yield the value  $+199^\circ$  (for an infinitely long chain). Starch, amylopectin, and amylose yield the value  $+200^\circ$  with a range of error  $\pm 2^\circ$  or more.

The existence of 1,6- $\alpha$ -glucosidic links in starch components can only be satisfactorily established by experiments involving methylation of the trisaccharides and tetrasaccharides obtained from far-driven amylolytic hydrolyses. Even this assumes that the limit dextrins are not products of resynthesis. Myrbäck claims to have established the existence of 1,6-linkages with the aid of such methods. On the other hand, Caldwell and Hixon, by similar means, have arrived at the conclusion that in starch and  $\beta$ -limit dextrin less than 1% of the linkages are 1,6-linkages (12). If the discoveries and conclusions of the Haworth-Hirst school are to be accepted, and the properties and high molar weight of amylopectin explained, some

3 or 4% of such linkages are required. It is a matter of such great importance that further attention to the problem is justified. Not only is our knowledge of the structure of starch dependent on it, but, economically, it is of importance in industry. For instance, hydrolysis of corn or other starches by amylases, followed by alcoholic fermentation by ordinary yeasts, does not produce the full theoretical yield of alcohol, because the limit dextrins are unfermentable by such yeasts. Previous hydrolysis by acids also leaves unfermentable matters, and there may well be some synthesis of Fischer's isomaltose and similar unfermentable bodies from glucose during acid hydrolysis. The complete theoretical yield of fermentable sugars from starch is rather difficult to secure. Clearly, a more complete understanding of the structure of starch components and their breakdown by amylases and glucosidases would be of value in industry.

(e) *Criteria of purity.* While it is probable that bacterial and animal amylases are  $\alpha$ -forms unadmixed with  $\beta$ -amylase, it cannot be assumed without evidence. There is no reliable evidence of an  $\alpha$ -amylase being separated into components exhibiting different amylolytic characteristics. The recorded attempts at fractional inactivation of presumed component enzymes have failed. Purification of pancreatic amylase by alcohol precipitation, dialysis, etc., gave rise to no great variation in the ratio of liquefying to saccharifying power (73). Much more recent work also supports the view that the starch-liquefying and dextrinizing activities are due to one and the same enzyme (7, 30). The early rapid production of  $\alpha$ -dextrins followed by the markedly slower breakdown of these dextrins to maltose, particularly in the case of malt  $\alpha$ -amylase, gives rise to the idea that only the first of these actions is due to the  $\alpha$ -enzyme and that the second might well be a function of relatively small quantities of  $\beta$ -amylase present. The mutarotational phenomena on record could be explained as follows: The maltose production is so slow that the  $\beta$ -mutarotation could not be observed, while in any case the much faster and overlapping production of  $\alpha$ -dextrins would mask part of it. Most mutarotation observations on  $\alpha$ -amylase fission products have been made before the rapidly formed  $\alpha$ -dextrins had finished their mutarotation. The slow, lingering saccharification lasting many days would be due to such small traces of  $\beta$ -amylase that the complete inactivation of these by heat, for instance, would be a matter of difficulty, even when the original preparation obviously contains an overwhelming excess of the  $\alpha$ -form, as *e. g.*, salivary amylase.

Criteria of purity, especially as regards malt  $\alpha$ -amylase, have usually consisted in the ratios of the amylase activities in their various manifestations.



Thus, Ohlsson (67) employed the ratio of velocity of increase in reducing power to velocity of disappearance of iodine coloration (using appropriate units) in his classical work on the amylases of malt. Blom (7) and co-workers extended and refined this method, using also the rate of fall in viscosity of a specially prepared starch paste. Aqueous extract of malt was heated in thin-walled vessels by immersion in water baths at 60° and 65° for periods of time and the above three activities were measured. The values of these rapidly fell during the first few minutes, while the ratios changed markedly. This was due to the relatively rapid destruction of the  $\beta$ -amylase, the predominant form in malt. However, after suitable intervals of time the ratios ceased to change beyond experimental error. The latter is rather large in some cases and the ratios are correspondingly affected. Such constancy of the ratios was taken to indicate that the  $\alpha$ -form alone survived the differential inactivation and was now pure. For ordinary purposes it is, but the destruction of the last traces of the  $\beta$ -enzyme may be long delayed. In performing experiments involving prolonged hydrolysis to prepare limit dextrins, a slight trace of the  $\beta$ -form lingering on may be a serious matter. The above experiments have recently been repeated by Hollenbeck and Blish (30), using different technique for liquefaction and dextrinization measurements, and by Myrbäck (61), omitting liquefaction but including observations on relative rates of dextrinization, saccharification, and glucose and maltose formation. Similar conclusions are drawn, and, in particular, that malt  $\alpha$ -amylase so prepared, *i. e.*, by Ohlsson's method, is a single enzyme responsible for all the functions, *viz.*, liquefaction, dextrinization, and maltose and glucose formation.

The  $\alpha$ -amylase of malt has sometimes been regarded as a type enzyme for reference and the ratios of other presumed  $\alpha$ -amylases compared with it. Certain difficulties appear, especially if liquefying power is being measured. The  $\alpha$ -amylases of different origins have different pH susceptibilities, temperature coefficients, and so on, and conditions including the buffer substances used must be selected to permit accurate measurement and comparison of two or more different amylases. Liability to inactivation is sometimes a source of difficulty. The differing values of the achromic point for various  $\alpha$ -amylases, quoted above, and other such observations have led to the opinion that many of the amylases contain both  $\alpha$ - and  $\beta$ -forms. Such a conclusion is perfectly sound when applied to malt, for example. Variation in affinities for starch components and dextrins as discussed above would go far to account for the variations observed among the better-known  $\alpha$ -amylases. Probably the determination of the Ohlsson-Blom ratios employing  $\beta$ -limit dextrin ( $\alpha$ -amylodextrin) as a substrate would be a refinement. The effect of a trace of  $\beta$ -amylase would be thrown into relief.

A further criterion of purity is that furnished by the Wijsman diffusion method (80). Appreciable quantities of  $\beta$ -amylase (but not traces) present in  $\alpha$ -amylase preparations can be measured by the method of Kneen and Sandstedt (42).

(f) *Erosion of starch granules.* The dissolution of starch in germination of grain, tubers, etc., is accomplished by enzymes present *in situ*; and it has usually been assumed that the amylases are solely responsible. Experiment showed, in the days of Brown (11), that what we now regard as  $\beta$ -amylase exercised virtually no such effect, but that  $\alpha$ -amylase (the "diastase of secretion" in germinating barley) brought about erosion and pitting. Microscopic observation strongly suggests that the outer or surface layer of the granule is much more resistant to amylases than the starch within. There is today very little doubt that the  $\alpha$ -amylase is solely responsible for the first "breakthrough" of an intact granule surface (41), undamaged by grinding, heating, or other processes.

### III. Influence of Ions on Activity and Stability of Amylases

Amylase substrates are but little ionized, and that mainly by virtue of phosphate. The amylases, on the other hand, in any stage of purification yet attained, appear to consist, in part, of protein material and to exhibit in their activities much the same response as proteins to variations in ionic concentrations, whether hydrogen or salt ions be concerned. The malt amylases, for instance, behave as if they are amphoteric. (While the  $\alpha$ -amylases exercise, qualitatively, the same specific actions on starch and its components, there are decided differences in their responses to ions, temperature, and such factors. For instance, the  $\alpha$ -amylases exhibit optimal activities as follows: malt  $\alpha$ -amylase, pH 5.3 in 0.015 *N* acetate buffer; pancreatic and salivary amylase, pH 6.8 in 0.005 *N* phosphate (25); and *Aspergillus* amylase, pH 5.0 (13)—all containing optimal concentration of sodium chloride. The bacterial amylases exercise optimal activities at pH 7.)

On the other hand, stability in the case of malt  $\alpha$ -amylase is optimal at pH 6.5–7 (67). Consequently, the appropriate value of pH in industrial processes, in which this enzyme is the one to be considered, will depend on the temperature. As this approaches 70° C. at which the enzyme is fairly rapidly inactivated, the most favorable reaction changes from pH 5.3 toward pH 7. The  $\beta$ -amylases of cereal grains exhibit optimal activities at and near pH 4.6, and optimal stabilities at about pH 6.

(In general, the effects of salts are consistent with their known effects on proteins, particularly globulins, namely, to increase solubility at suitable values of pH. Many of the substances which have been claimed as amylase activators, complements, and so on, are apparently merely salts functioning in this way, influencing the activity chiefly by increasing solubility,

dispersion, or stability of the enzyme. Generally, the more the enzyme is purified, the more unstable it becomes. )

(Salivary amylase is inactive in the absence of salts, but 0.05 *M* sodium chloride fully reactivates the enzyme. The latter (or its "colloidal carrier") seems, in this connection, to be of the nature of a globulin with its isoelectric point near pH 4)(66). Liver and pancreatic amylases, also, are inactive on dialysis. On the other hand, chlorides increase the activity of malt amylase ( $\alpha + \beta$ ) on each side of its optimum pH, 4.6, but not at the latter reaction. In effect, the optimal zone is widened (74). The effect in this case, however, is very much smaller than with the animal amylases. *Aspergillus* amylase is not apparently influenced by sodium chloride, but its pH optimum is altered to pH 5.1 by the presence of salts of fatty acids at 30° C. (4). This enzyme and  $\beta$ -amylase also have their activities depressed on the acid side of the optimal pH by anions of fatty acids (4). (Chloride and other ions alter the shape of the pH-activity curves of pancreatic and salivary amylases)(55). Buffers often exert an effect on the pH optimum and on the activity of the enzyme at other values of pH, so that the nature of salts present should be taken into account as well as pH, when enzymic activity is to be under control in industrial processes, analytical procedure, and research operations.

(The stability of an enzyme, as well as the activity, is often influenced. The calcium ion increases the stability of  $\alpha$ -malt amylase (44), pancreatic, and other  $\alpha$ -amylases, but decreases that of  $\beta$ -amylase of barley. Certain salt ions inactivate amylases irreversibly, mainly by virtue of poisoning effects. Among these are iodides (31) (possibly through the liberation of traces of free iodine), fluorides, heavy metals, and the usual protein precipitants. There is also reversible inactivation of  $\beta$ -amylase by certain oxidants such as peroxides, iodine, and by substances, as yet ill defined, which occur in many grains (14, 43). Ascorbic acid, which is present in barley extracts, inactivates both the  $\beta$ - and  $\alpha$ -amylase of malt. On the other hand,  $\beta$ -amylase (from barley) is "activated" by hydrogen sulfide, although the mechanism whereby this is achieved is uncertain)(14).

#### IV. Stability of Amylases to Heat

As is generally true of hydrolytic enzymes, dry preparations are much more stable to heat than aqueous solution. In the latter form, the purer the preparation, the more readily inactivation takes place. In the presence of substrate with which amylases combine, stability is increased. For

these reasons a purified preparation of  $\alpha$ - or  $\beta$ -malt amylase would be more completely inactivated at, say, 60° C. in ten minutes, than its equivalent in a cold aqueous extract of malt, which in its turn would survive the ordeal less successfully than the corresponding enzyme contained in malt mashed with water, as in a brewery. (For a long time it was not realized why the starch conversion in a brewery mashing process was a more complete one than that achieved by cold water extract of malt added to starch paste in equivalent quantities and under the same conditions of temperature, etc. In the former case—neglecting a certain amount of amylase released by proteolytic action only—the amylases survive longer because of a number of factors, concentration of substrate being one. The optimum temperature for saccharification in a malt mash will depend on the duration of the mash, its concentration, pH, the proportion of  $\alpha$ - and  $\beta$ -amylases in the malt chosen, and on a number of purely technical factors such as the method employed for mixing the grist with the water.) Infusion at about 65° C. leaves mainly  $\alpha$ -amylase surviving in the unboiled wort. In distilleries and malt vinegar factories, the wort is not boiled but is fermented directly. During this process more dextrans are broken down by the surviving amylases to fermentable sugars and fermented away, the more complete the fermentation, the higher the yield of alcohol or acetic acid. For this amyolytic hydrolysis to give the most complete conversion before fermentation is finished, *i. e.*, in a few days at ordinary temperatures, it is desirable that not merely  $\alpha$ -amylase, but some  $\beta$ -amylase, should survive the mashing process of, probably, two hours' duration. This is one reason why distillers and vinegar manufacturers mash their grists at lower temperatures, *e. g.*, 60°, than those finally attained in the mash by infusion brewers, the latter being concerned with the total yield of extract as well as its fermentability. Indeed, in brewing, there should be some unfermentable carbohydrate left behind in the finished product, amounting to perhaps 35–40% of all that originally passed into solution. ✓

The stability of the two malt amylases to heat is completely recorded (44, 67) in so far as cold-water malt extract is concerned. It is, however, of little value to give temperatures of complete inactivation for reasons already given. In general,  $\alpha$ -amylases, particularly bacterial, withstand heat better than  $\beta$ -amylases, a fact which is of use in industry, for example, in the manufacture of dextrans by enzyme action.

(Amylases are inactivated, in general, by conditions which favor protein denaturation and coagulation. These include surface activity and agitation.) Holmbergh (33) even utilizes differential inactivation of the malt amylases by such means to purify them, the  $\alpha$ -form being the more stable.

## V. Kinetics of Amylase Action

Hydrolytic enzyme action may take place under either of two main conditions, but may pass through more than one of these before completion. They may be indicated briefly as follows:

(1) *Substrate is present in excess.* Velocity of reaction is proportional to enzyme concentration and remains constant as long as the latter is unaffected by inactivation or combination with reaction products. Ultimately the approaching exhaustion of the substrate brings condition (2) into operation.

(2) *Enzyme is present in excess.* Velocity of reaction is usually monomolecular and depends on both enzyme and substrate concentrations. Two complications may arise: (a) If the initial substrate concentration is such that condition (1) holds for only a short time, the period of overlap between (1) and (2) may make it difficult to recognize the conditions. (b) If the reaction under condition (1) takes place under circumstances conducive to inactivation of the enzyme, *e. g.*, at a high temperature, this inactivation may be monomolecular—as is, often, the denaturation of proteins—and so the progress of the reaction may simulate condition (2).

Alpha-amylase acts on a succession of substrates. Its low affinity constant, about  $K$ , 0.024 (expressed as per cent) (16), with starch means that as long as the concentration of unchanged starch is above, for example, 0.25%, condition (1) holds (16). However, the affinity for dextrans is much less, and when all starch has disappeared, the solution may need to be very much more concentrated in respect to dextrans for condition (1) to continue to hold. This is one reason why the course of  $\alpha$ -amylase hydrolysis exhibits a more marked bend or inflection in the progress—time curve when dilute starch solutions are employed. The fall in reaction velocity at this point is due not only to the decreased affinity for substrate, but also to the onset of an apparently monomolecular stage of the reaction.

Evidently, with concentrated starch systems, as in brewery mashes, the  $\alpha$ -amylolytic action will continue under condition (1) for most of the course of the reaction, *i. e.*, relatively more starch and dextrin will be hydrolyzed by the  $\alpha$ -enzyme than with a corresponding dilute system.

Beta-amylase cannot operate under condition (2) until the reaction approaches the limit, since the molecular concentration of the substrate remains unchanged for most of the reaction. Actually, irrespective of the initial starch concentration, the velocity is constant until 35–40%  $R_m$  value is attained (39, 52).

When the two enzymes act simultaneously, two main possibilities arise:

- (1) Substrate is in excess and the velocities of reaction, promoted by the two enzymes (as measured by reducing power), are additive (18, 42, 77).  
(2) Enzymes are in excess, and competition for the substrate ensues.

Since  $K_s = 0.3$  (expressed as per cent) for malt  $\beta$ -amylase (52) and starch, and about 0.024 for malt  $\alpha$ -amylase (16), one would expect the  $\alpha$ -enzyme to act on the starch 0.3/0.024, or about twelve times faster than the  $\beta$ -enzyme. The truth of this statement is borne out by the recorded determinations of affinity constants for malt amylase and starch.

The values (expressed as per cent) 0.048 (48) [malt amylase (Merck), Lintner starch at 20° C., and pH 5] and 0.078 (21) [Lintner starch, 25° C., and pH 4.7] have been recorded, each approximating much more nearly the value for the  $\alpha$ - than for the  $\beta$ -enzyme, and indicating that the  $\alpha$ -amylase dominated the early stages of the reaction. However, with the disappearance of starch from the reaction medium, the  $\beta$ -amylase tends to take charge. Accurate values for the affinity constants of the enzymes with the dextrins are not yet available. Inactivation of the enzymes at different rates introduces a complication and this often takes place, especially in technical processes. Clearly, when the two amylases act simultaneously the result will vary widely with conditions of temperature and pH, as well as with the relative activities of the two enzymes present at the outset. Competition for the substrate favors the  $\alpha$ -amylase in the case of malt; so do high temperatures at ordinary values of pH (67), the calcium ion (44), and agitation (33). On the other hand, if conditions permit the survival of the  $\beta$ -enzyme until dextrinization is complete, it can compete successfully with the  $\alpha$ -amylase and saccharify most of the dextrins, a process which is likely to be much more rapid than saccharification of the dextrins by the  $\alpha$ -enzyme. This presumably happens in the mashing process in breweries, etc., in which almost all the malt starch is "converted" in a few minutes. It should be remembered that ordinary commercial brewers' and distillers' malts owe much more of their saccharifying power to  $\beta$ - than to  $\alpha$ -amylase. The relative activities of the  $\alpha$ - and  $\beta$ -amylases in malt—each expressed as saccharifying power—vary, of course, particularly with kilning and curing conditions. For American brewers' malts, an average of 85% of the saccharifying activity may be ascribed to the  $\beta$ -amylase (41, 42). The  $\beta$ -mutarotation, observed by Kuhn with malt amylase alone, is evidence of this, the  $\beta$ -amylolytic action prevailing, notwithstanding the greater affinity of the  $\alpha$ -component for the initial substrate, starch.

The  $\alpha$ -amylase in various forms now on the market is much used in industry, for instance, in the desizing of fabrics, the manufacture of dextrin, and the liquefaction of

starch for sizing and of mashes for alcohol manufacture. In the manufacture of dextrans the starch is suspended in cold water, and the enzyme added and mixed in. The slurry is heated and agitated until gelatinization and the desired degree of dextrinization have been attained. In this, allowance is made for further enzyme action that will take place during the subsequent process of heating to a still higher temperature, to destroy the enzyme. Since gelatinization occurs most readily in alkaline reactions an  $\alpha$ -amylase with a relatively alkaline pH optimum and high heat stability is best, and clearly, bacterial amylases are indicated. For desizing, malt amylases and fungal amylases also may be used, for there is no objection to saccharification as well as dextrinization taking place.

We may conclude this survey with a generalization. The  $\alpha$ -amylase is the fundamental amylase, with its high affinity for starch (even starch granules) and its powers of relatively rapid dextrinization. As is the case with malt  $\alpha$ -amylase, it can ultimately catalyze the reaction by itself as far as it would in the presence of the  $\beta$ -enzyme. The latter, acting in conjunction with the  $\alpha$ -amylase, fulfills the role of an accessory which speeds up and completes the process of saccharification. Its affinity for starch is less, for dextrans greater, than those of the  $\alpha$ -enzyme. If the  $\alpha$ -amylases had been well understood before the existence of  $\beta$ -amylase was known, and the latter first encountered only in the presence of the former, it might well have happened that the  $\beta$ -amylase would have been mistaken for an activator or complement of the  $\alpha$ -enzyme, since its functions are so largely complementary.

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# THE AMYLASES OF WHEAT AND THEIR SIGNIFICANCE IN MILLING AND BAKING TECHNOLOGY

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## I. Introduction

The importance of amylases, the enzymes which catalyze the hydrolytic degradation of starch, in the production of alcoholic beverages early stimulated the interest of scientists, and a vast body of knowledge now exists on their occurrence, identification, properties, and evaluation. Payen and Persoz (185), in 1833, applied the name diastase to the starch-splitting enzymes of barley malt and this designation has persisted until recent times. The term amylase is now preferred since it conforms to the modern system of enzyme nomenclature. As early as 1879, Märcker (159) concluded from thermal and acid inactivation studies that there were two diastatic enzymes in malt. Lintner (148), in 1887, confirmed this observation and concluded that whereas malt contained a starch-liquefying and a starch-saccharifying enzyme, ungerminated barley contained only the latter component. This distinction between germinated and ungerminated cereals was expanded and clarified by many subsequent workers. Although there is no general agreement as to the number of enzymes which are involved in the hydrolytic degradation of starch, two main types are now recognized, namely,  $\beta$ -amylase, the so-called saccharogenic amylase, and  $\alpha$ -amylase, the dextrinogenic and liquefying amylase. The extensive literature on the amylases has been reviewed by Hanes (88), Blish, Sandstedt, and Kneen (23), Caldwell and Adams (42), and others, but a brief summary of our present knowledge of their nature and properties will be presented here to serve as a basis for a discussion of the amylases of wheat and flour and the significance of amylase activity in milling and baking technology.

## II. Occurrence and Properties of the Amylases

### 1. $\beta$ -Amylase and $\alpha$ -Amylase

Beta-amylase predominates, and occurs abundantly, in ungerminated cereal grains. It is highly inactive on raw, native starch and brings about the hydrolysis of only the starch which has been rendered susceptible by mechanical injury, by treatment with acid, or by gelatinization. Its action is characterized by the immediate appearance of maltose (which is liberated as  $\beta$ -maltose) without any marked change in viscosity, so that the enzyme must split off successive terminal maltose units from the starch molecule (36, 37, 71). Hydrolysis ceases when approximately 60% of the theoretical yield of maltose is obtained and there remains a virtually non-reducing dextrin of high molecular weight which gives a blue-violet or violet color with iodine. This residual dextrin was noted by early workers

and was called erythrogranulose by Wijsman (238), and  $\alpha$ -amylodextrin by Baker (14). In addition to maltose, traces of glucose have been reported upon the prolonged action of  $\beta$ -amylase on soluble starch (87). As pointed out by Caldwell and Adams (42), maltose is the sole significant product of low molecular weight which is formed by  $\beta$ -amylase; the traces of glucose may arise as a result of the presence of a contaminating enzyme or may represent residual glucose molecules after maltose has been split from glucosidic chains with an uneven number of glucose units.

Recent advances in our knowledge of the structure of starch afford an explanation for the formation of the residual dextrin. Although the heterogeneous nature of the common cereal and tuber starches has long been recognized, methods have only recently been perfected which provide a clear-cut separation into two components to which the names amylose and amylopectin, earlier used in a different sense, have been applied by most investigators. The accumulated evidence of many investigators, which has been summarized by Meyer (167), Kerr (121), Hassid (92), and others, indicates that amylose consists of unbranched chains of glucose residues, joined only through  $\alpha$ -1,4-glucosidic linkages. Amylopectin, on the other hand, possesses a highly ramified structure in which the branches are formed by  $\alpha$ -1,6-glucosidic linkages. Meyer concludes from the available evidence that, on the average, branching occurs at every twenty-fifth glucose unit and that the outside branches "consist of 15 to 18 glucose units, whereas the inside parts of the chains between branched positions are about 8 to 9 units long." The linear component, which can be selectively precipitated from autoclaved starch solutions with butyl or amyl alcohol (203) or with higher fatty acids (204), comprises 25 to 30% of the common starches. Under suitable experimental conditions,  $\beta$ -amylase completely hydrolyzes amylose to maltose (93, 167), the maltose units being split off from the nonaldehydic end of the chain (87, 174, 179). Amylopectin, however, yields a high molecular weight dextrin with  $\beta$ -amylase and it is assumed that the  $\beta$ -amylase attacks the nonreducing ends of the free branches of the starch molecule and that its action stops when the  $\alpha$ -1,6-glucosidic linkages are reached (167, 173). The unhydrolyzed residue from amylopectin thus comprises the residual dextrin which is formed when  $\beta$ -amylase acts on gelatinized starch.

Alpha-amylase occurs along with  $\beta$ -amylase in germinated cereals and comprises the amylase of animal fluids and of certain bacteria and fungi.

In contrast to the action of  $\beta$ -amylase,  $\alpha$ -amylase brings about a marked decrease in the viscosity of starch pastes, and rapidly cleaves the starch to products which do not give a color with iodine; there is little production of

maltose during the early stages, and that which is liberated is the alpha form (72, 179). When the achromic stage is reached, the reducing power of the products of the action of malt  $\alpha$ -amylase on starch pastes corresponds to about 30% of the theoretical yield of maltose (88, 122, 195). Further hydrolysis proceeds at a much slower rate to final reducing values which vary with the enzyme concentration. The dextrins hydrolyze to maltose for the most part, with some glucose, together with other reducing carbohydrates among which isomaltose, tri- and tetrasaccharides have been reported (147, 173, 174, 182). Amylose and also amylopectin are rapidly split by  $\alpha$ -amylase, and hydrolysis of the latter does not result in the formation of a resistant dextrin of high molecular weight. Thus, in contrast to  $\beta$ -amylase which splits off maltose from the ends of the starch chains,  $\alpha$ -amylase appears to effect an internal disintegration of the molecule and thereby brings about rapid liquefaction with the production of dextrins, rather than maltose.

The joint action of  $\alpha$ - and  $\beta$ -amylases brings about much more rapid hydrolysis of starch pastes than either enzyme alone. The cleavage brought about by  $\alpha$ -amylase produces a new point of attack for the action of  $\beta$ -amylase. The products formed by each enzyme are subject to further hydrolysis by the other and a high degree of hydrolysis is obtained without the accumulation of a resistant dextrin, as in the instance of  $\beta$ -amylase action, or a marked decrease in rate, as is observed with  $\alpha$ -amylase after the achromic point is reached. When both enzymes are present, the achromic stage is reached more rapidly than when  $\alpha$ -amylase acts alone.

In common with other enzymes, the optimum hydrogen ion activities of  $\alpha$ - and  $\beta$ -amylase depend upon a number of factors such as purity of the preparation, enzyme and substrate concentration, nature of the electrolytes which are present, the temperature, and the length of time the reactions are carried out. It is, therefore, not surprising that different optima are to be found in the literature. Creighton and Naylor (52) found that wheat  $\alpha$ -amylase exerted its optimal activity at pH 4.9 to 5.3 when acting upon soluble potato starch in 0.02 to 0.06 *M* phosphate buffer for 30 minutes at 40° C.; employing 1% starch, but otherwise similar conditions, wheat  $\alpha$ -amylase exhibited optimal dextrinogenic activity between pH 4.6 and 6.3. In studies of the influence of ten buffers of constant ionic strength on the activity of wheat  $\beta$ -amylase, as measured with 1% starch at 30° C., Ballou and Luck (16) found that the optimal activities varied between pH 4.5 and 5.3, depending upon the buffer; a similar range, but shifted slightly to the alkaline side, was noted for taka-diastase (an  $\alpha$ -amylase).

Alpha- and  $\beta$ -amylase differ markedly in their stability to high hydrogen ion activities and to temperature, especially in crude aqueous extracts. Beta-amylase is much more thermolabile than  $\alpha$ -amylase, whereas  $\alpha$ -amylase is the more sensitive to high hydrogen ion activities. These differences, which were noted by early workers, were utilized by Ohlsson (178, 179) for separating the two amylases of barley malt. According to his technique,  $\beta$ -amylase is inactivated by heating the extract at a pH of approximately 7.0 for 15 minutes at 70° C., while much of the  $\alpha$ -amylase activity will remain. On the other hand, if the extract is acidified to pH 3.3, held at 0° C. for 15 minutes, and then neutralized, most of the  $\alpha$ -amylase will be inactivated with but little loss of  $\beta$ -amylase. These procedures have been widely used for preparing the two amylases from malted grain. Kneen, Sandstedt, and Hollenbeck (133) recently studied the differential stability of the amylases of wheat and barley malt to temperature and acidity from the viewpoint of isolating "pure" preparations. The stability of either amylase was influenced by both temperature and acidity, although temperature was the more effective in inactivating  $\beta$ -amylase, and high hydrogen activity was the more effective in inactivating  $\alpha$ -amylase. The presence of calcium ions was found to increase the thermolability of  $\beta$ -amylase and decrease that of  $\alpha$ -amylase. Similarly, the presence of calcium ions decreased the loss of  $\alpha$ -amylase and increased the loss of  $\beta$ -amylase at high hydrogen ion activities. These observations were employed in developing an improved Ohlsson technique for preparing  $\alpha$ - and  $\beta$ -amylase from malt extracts. This technique proved to be more efficient than methods of preparation which had been developed by previous workers, based on fractional precipitation with ammonium sulfate and differential solubility in alcohol.

## 2. Other Starch-Degrading Enzymes

All investigators do not agree that the starch-degrading properties of grain and malt can be ascribed entirely to the action of  $\alpha$ - and  $\beta$ -amylase. In 1890, Brown and Morris (37) postulated the existence of a cytolytic enzyme in barley malt which functioned in hydrolyzing the outer, and supposedly very resistant, covering of native starch granules. More recently, Blish, Sandstedt, and Mecham (24) obtained evidence which they interpreted as indicative of the existence of a "raw starch factor" in normal wheat flour which was involved in the slow hydrolysis of raw starch upon the autolysis of flour suspensions. However, in a later study, Kneen, Beckord, and Sandstedt (128), with more refined techniques, found that the ability of malt to hydrolyze raw wheat starch was highly correlated with

$\alpha$ -amylase activity. This action is now quite generally attributed to  $\alpha$ -amylase (41, 127, 221).

Some investigators have believed that the liquefaction of starch is, in part at least, independent of both dextrinization and saccharification (44). This view was strengthened by Waldschmidt-Leitz and Mayer (232) who reported the isolation of an enzyme from barley malt which liquefied starch pastes with only slight dextrin or sugar formation. The enzyme was called *amylolphosphatase* because its liquefying action was attributed to the hydrolytic cleavage of phosphoric acid from a presumed starch phosphoric acid ester. The evidence which led to the postulation of this enzyme has been summarized by Mayer (165). That liquefaction is largely independent of saccharification and dextrinization was indicated by treatment of starch with iodine, which greatly reduced saccharification, and by heat inactivation of the saccharogenic amylase, which markedly decreased dextrinization; both treatments had little effect upon liquefaction. On the other hand, other workers believe that liquefaction is due chiefly to the activity of  $\alpha$ -amylase. This view is supported by the studies of Hollenbeck and Blish (101) who showed that a close parallelism existed between the  $\alpha$ -dextrinizing and the liquefying activities of three amylase preparations from widely different sources (malt, bacterial, and fungal) and that the two types of activity were equally affected by heat and by changes in hydrogen ion activity. In a more recent study by Redfern and Landis (190) of the relative liquefying and dextrinizing activities of  $\alpha$ -amylases from malt, bacteria, and mold, it was found that the liquefaction rate curves for equivalent  $\alpha$ -dextrinizing amounts of the three types of amylases were not superimposable. However, their data show that either the liquefying or dextrinizing method may be used as a measure of  $\alpha$ -amylase activity within any one type. This is also shown by the results of a collaborative study of the  $\alpha$ -dextrinizing and the liquefying activities of a series of malts reported by Dickson (58).

In addition to the amylases, starch is degraded by other enzymes to which only passing reference will be made here. Kneen (126) has recently reported the presence of a glycosidase in sorghum malt which supplements the starch-degrading actions of  $\alpha$ - and  $\beta$ -amylase. The researches of Cori and Cori (51) and of Hanes (89) have established the existence of phosphorylases in certain plant and animal tissues. In the presence of inorganic phosphates and under suitable conditions, phosphorylases catalyze the formation of glucose-1-phosphate from starch or glycogen. In the presence of traces of a suitable polysaccharide, they also catalyze the synthesis of a starch-like or glycogen-like material from glucose-1-phosphate.

As first reported by Schardinger (202), the amylase of *Bacillus macerans* has the distinctive property of hydrolyzing starches to a mixture of two characteristic nonreducing crystalline dextrans which possess a closed ring structure. The literature on the phosphorylases and on the amylase of *B. macerans* has been adequately reviewed by Caldwell and Adams (42).

### III. Measurement of Amylase Activity

#### 1. General Principles

The saccharifying property of  $\beta$ -amylase and the liquefying and dextrinizing properties of  $\alpha$ -amylase are utilized in measuring their activities. As raw starch is not attacked by  $\beta$ -amylase and only slowly by  $\alpha$ -amylase, the starch substrates are prepared in the form of pastes or solutions. Substrates prepared by the heat gelatinization of starch are not sufficiently homogeneous to give satisfactorily reproducible results in amylase activity measurements, and starches which have been rendered soluble by treatment with acid, according to the procedure of Lintner (148), or by mechanical treatment are usually employed. Starches rendered soluble by mechanical means are used particularly for viscometric measurements of liquefying activity (26, 114, 115, 232).

In saccharifying and dextrinifying procedures which are designed to secure a measure of amylase concentration, the enzymes are extracted from the material under study by means of a suitable solvent and their action on the starch substrate measured (under standard conditions of temperature, pH, and other factors) in such a way that the results will be proportional to the enzyme concentration. This may be accomplished in two ways: (1) by determining the enzyme concentration or the time required to bring about the conversion of a constant fraction of the substrate; and (2) by measuring the amount of hydrolysis which occurs under conditions in which the extent of conversion is a linear function of the enzyme concentration. The procedure involving a constant amount of hydrolysis is exemplified by the well-known Wohlgemuth method (239) for  $\alpha$ -amylase; the enzyme dilution required to bring about a definite amount of hydrolysis in a given time is determined and the enzyme strengths of different preparations are obviously directly proportional to the dilutions. With procedures in which the extent of the hydrolysis of the substrate varies, several methods of calculation have been evolved which relate the extent of hydrolysis to the enzyme activity.

It has been adequately demonstrated that  $\alpha$ -amylase contributes to the saccharification brought about by malt; similarly,  $\beta$ -amylase contributes to the dextrinizing activity. Hence, with mixtures of the amylases, determinations of the saccharogenic and dextrinogenic activities are not true measures of their respective  $\beta$ - and  $\alpha$ -amylase activities. Differential destruction of the amylase components is not sufficiently quantitative to



be applicable to this problem, but recently methods have been developed which permit the estimation of  $\alpha$ - and  $\beta$ -amylase in mixtures.

In addition to methods designed to measure enzymic strength, autolytic procedures are widely used in products control; such methods measure the summation of several factors such as enzyme concentration, substrate susceptibility, and any other variables which may be operative, so that they give an integrated result of practical utility (136).

The methods which are frequently employed in amylase studies of malts, wheats, and flours will be briefly outlined.

### 2. $\alpha$ -Amylase Activity

Dextrinogenic activity is usually determined by the Wohlgemuth procedure (239) or one of its modifications (90, 201, 210). This involves the hydrolysis of a buffered starch solution at a constant reaction temperature until a definite stage is reached. Many investigators have selected the achromic stage (at which no color is given with iodine) as the end point, but greater precision in visual color comparison is obtained if an earlier stage of hydrolysis is selected. Sandstedt, Kneen, and Blish (201) use a commercial dextrin which gives a red-brown color with iodine as a color standard. Since the Wohlgemuth values as commonly determined with an amylase mixture are indicative only of the combined dextrinizing activity of  $\alpha$ - and  $\beta$ -amylase, these workers render the method specific for  $\alpha$ -amylase by adding an excess of  $\beta$ -amylase to the reaction mixture. This procedure is based on their finding that increases in the ratio of  $\beta$ - to  $\alpha$ -amylase in the reaction mixture increased the rate of dextrinization up to a point beyond which further increments of  $\beta$ -amylase had essentially no effect. For the determination of low  $\alpha$ -amylase activity, as in wheat flour, Kneen, Sandstedt, and Hollenbeck (133) have developed a microdextrinization procedure. This involves prolonged digestion of soluble starch with the flour extract in the presence of excess  $\beta$ -amylase followed by complete dextrinization with a malt or  $\alpha$ -amylase solution of known dextrinizing activity. From the resulting data, the dextrinization due to the small quantity of  $\alpha$ -amylase present in a flour extract may be calculated. Hanes and Cattle (90) have employed a spectrophotometric procedure to follow the changes in iodine coloration during dextrinization.

Liquefying activity of  $\alpha$ -amylase is determined by measuring the decrease in the viscosity of reaction mixtures (26, 114, 115, 232). Viscometric methods are influenced by a number of factors, but if starches rendered soluble by chemical or mechanical means are used and care is taken to standardize other conditions, reproducible measurements may be made.

### 3. $\beta$ -Amylase Activity

In preparations free, or relatively free, from  $\alpha$ -amylase,  $\beta$ -amylase may be determined by measuring its saccharogenic activity on soluble starch under standard conditions. Modifications of the Lintner method (148), proposed by Anderson and Sallans (6) and by Kneen and Sandstedt (131), are now commonly employed in malt and flour studies in which maltose is measured by a ferricyanide method.

Kneen and Sandstedt (131) have developed a useful quantitative method for the determination of  $\beta$ -amylase in wheat and barley malts, that is, in the presence of  $\alpha$ -

amylase. They found that the saccharogenic activities of the two enzymes are additive over a rather wide range in relative concentrations. The  $\alpha$ -dextrinogenic activity is determined by the method of Sandstedt, Kneen, and Blish (201) already outlined and the value converted to its equivalent in saccharogenic activity by reference to a table expressing the relation between  $\alpha$ -dextrinogenic and  $\alpha$ -saccharogenic activity, as determined with an  $\alpha$ -amylase preparation. The  $\beta$ -saccharogenic activity is then calculated by subtracting the  $\alpha$ -saccharogenic value from the total saccharogenic activity.

#### 4. Autolytic Methods

Autolytic methods have long been used in wheat flour technology to secure a measure of amylase activity under conditions which approximate those encountered in dough. In 1907, Wood (240, 241) recognized that the sugar formed by diastatic action is of importance in relation to gas production, particularly during the later stages of dough fermentation. He proposed two tests, one of which involved the measurement of the sugars produced upon autolysis of a flour-water suspension for three hours at 30° C., conditions which simulate the time and temperature of dough fermentation. The other method comprised the measurement of the carbon dioxide produced by a dough made with yeast, flour, and water. Swanson and Calvin (228) studied various factors influencing the autolytic sugar production in aqueous flour dispersions. This work was extended in 1922 by Rumsey (194), who developed a standard autolytic procedure and expressed diastatic activity as the milligrams of maltose produced in one hour at 27° C. in a dispersion with a flour to water ratio of 1 to 10. Although Rumsey established that the optimum activity was manifested at pH 4.7, he refrained from employing buffers since "any practical method for the measurement of the value of diastatic enzymes in baking must be based upon conditions as they exist in the dough." The original Rumsey method has been improved and greatly simplified through control of the acidity at pH 4.7 (152), elimination of the blank, and the use of a more convenient method for determining the reducing sugars present after autolysis at 30° C.; these improvements are embodied in the method described by Blish and Sandstedt (20), in which the determination of maltose is based upon the ferricyanide method of Hagedorn and Jensen (86). Various other methods for determining the reducing sugars have been employed. Geddes and Eva (76) carried out a comparative study of the Blish and Sandstedt (20) ferricyanide method with the copper reduction procedure of Quisumbing and Thomas (188). They found that the former method gave slightly lower values, but was preferable to the latter from the standpoints of greater convenience, lower experimental error, and better differentiation between flours.

Later, Davis (55) organized a collaborative study in which the Blish and Sandstedt (20) ferricyanide method was compared with the copper reduction procedures of Bertrand (18) and Schoorl (205). These methods were rated in the following order of preference: 1, Schoorl; 2, Blish and Sandstedt; and 3, Bertrand. The Schoorl method gave a greater uniformity between laboratories and a greater differentiation between flours, although the experimental error was higher than that of the Blish and Sandstedt method. The Bertrand procedure was the least desirable, primarily because of its high experimental error. Polarimetric methods (35, 80, 186) have been proposed for estimating the amount of reducing sugars formed, but they do not appear to have come into wide use. Since the values obtained upon the autolysis of flour suspensions are influenced by several factors besides amylase content, such as variations in flour granulation, original reducing sugar content, and starch susceptibility, the data are now

usually designated as "maltose values" or "maltose figures." Kent-Jones and Saxby (120) prefer to express the results in "per cent units" instead of the rather inconvenient designation "milligrams of maltose per 10 g. of flour."

European workers have devoted considerable attention to the development of convenient tests for the detection of excess  $\alpha$ -amylase activity. For this purpose, Molin (169) determined the refractive index of the solution obtained after autolysis of a flour-water suspension at 62° C., a temperature which favors dextrin formation by  $\alpha$ -amylase. Certain modifications of this technique were introduced by Munz and Bailey (171).

Another autolytic procedure, which is based on the rapid liquefaction which accompanies the action of  $\alpha$ -amylase on starch paste, has recently been introduced. In 1937, Brabender (29) described a recording torsion viscometer, known as an amylograph, which provides a continuous automatic record of the changes in the viscosity of a flour-water suspension as the temperature is increased to 95° C. at a uniform rate. The increase in viscosity which takes place upon gelatinization of the starch is opposed by the liquefying action of the amylase present, and the height of the curves at maximum viscosity is an index of the liquefying activity (31). Anker and Geddes (8) have indicated that the amylograph can readily be used to measure the relative liquefying activity of amylase preparations by employing a common source of starch.

Modifications of the technique originally proposed by Wood (241) for the measurement of gas production in fermenting doughs are widely used in cereal laboratories. As the object of the test is to determine the inherent capacity of the flour for gas production (that is, the "gassing power"), such conditions as flour-water-yeast ratio, temperature; and time approximate those in dough fermentation; supplementary ingredients such as salt, shortening, sugar, and oxidizing improvers are normally omitted. The gas produced may be measured volumetrically, as by the procedure of Bailey and Johnson (11) and other devices (60, 94, 104, 113, 124, 135, 160, 168, 208, 212); manometrically by the widely used apparatus of Sandstedt and Blish (197) or its modification (154); gravimetrically (61), or by the use of the Brabender fermentograph (28). With the fermentograph, the doughs are placed in thin rubber balloons and suspended in a constant temperature bath from a balance; as gas is produced, the buoyancy increases and the gas production is automatically recorded. Eva, Geddes, and Frisell (63) found that the volumetric, manometric, and fermentograph methods gave results which were in close relative agreement.

#### IV. Amylases of Wheat

##### 1. *Amylases of Sound and Germinated Wheats*

Sound wheat contains relatively large amounts of  $\beta$ -amylase, but the content of  $\alpha$ -amylase is extremely low. Upon germination, there is a marked increase in the saccharogenic activity and  $\alpha$ -amylase activity also becomes very pronounced. The increase in amylase activity which occurs upon the germination of barley and other cereal grains was attributed by early investigators to the actual synthesis of enzymes. Ugrumow (231) found that both  $\alpha$ - and  $\beta$ -amylases were present in the early stages of development of wheat kernels; as the maturation progressed, the  $\alpha$ -amylase

activity disappeared, whereas the  $\beta$ -amylase activity decreased. Upon germination, the reverse process was noted;  $\alpha$ -amylase activity developed early and the  $\beta$ -amylase activity increased approximately 100%. Chrzaszcz and Janicki (45) showed that the sequence of changes in amylase activity during ripening and subsequent germination also occurs in barley and other cereals. Ford and Guthrie (69, 70) first demonstrated that proteolytic enzymes increased the saccharogenic activity of ungerminated barley and wheat, and the researches of several investigators (46, 149, 150, 175, 233, 235) have led to the conclusion that the increase in amylase activity coincident with the germination of barley is largely the result of the liberation of "bound" or latent amylase of the ungerminated cereal by the action of the proteolytic enzymes simultaneously developed during germination.

Several attempts have been made to explain the increase in amylase activity upon germination. Waldschmidt-Leitz and Purr (233) presented evidence to show that the increase may be caused by the formation in germinated barley of an amylase activator, *amylokinase*, but other workers (150) failed to confirm their results. Chrzaszcz and Janicki (46) postulated the presence of an amylase inhibitor, *sisto-amylase*; on germination of barley, the protein degradation products counteract the effect of the *sisto-amylase* and restore the full activity of the amylases. They reported that the action of the inhibitor could be counteracted by *eleuto substances* such as peptone or sodium chloride. In support of this theory, Janicki (109) has reported that the activation of amylase requires the action of reducing agents like hydrogen sulfide. Oxidized ascorbic acid functions as an amylase inhibitor and hydrogen sulfide converts it into the reduced form. He points out that there is sufficient copper present in barley to accelerate the oxidation of ascorbic acid.

This viewpoint has been attacked by Myrbäck and Örtenblad (176) and Hills and Bailey (99, 100), who favor the view that  $\beta$ -amylase is inactivated by being partly bound to protein and that it is released when the proteins are degraded by protease action. Myrbäck and Örtenblad (176) have shown that the barley proteases are activated by hydrogen cyanide and hydrogen sulfide, and this would explain the increase in amylase activity observed by Janicki (109) with hydrogen sulfide.

Hills and Bailey (99) confirmed the role of protease in increasing  $\beta$ -amylase activity in barley, but they found that the increase in  $\alpha$ -amylase activity during germination was due to an actual increase in the amount of the enzyme. They demonstrated that peptone activated a purified  $\alpha$ -amylase preparation equally as much as it did a malt extract, and it may be

assumed that the latter would be much richer in the sisto-substances postulated by Chrzaszcz and Janicki. That the total amount of  $\beta$ -amylase does not increase during germination, but is merely liberated from an inactive state in which it is bound to protein, and that the  $\alpha$ -component actually increases in quantity, is supported by other workers (54, 146, 180, 217).

These researches have been applied in the development of methods for determining the "free" and "bound" amylase in cereal grains. The total amylase activity is determined after digesting a suspension of the ground cereal with papain; subtraction of the "free" amylase activity, as determined without papain treatment, from the total value gives the "bound"

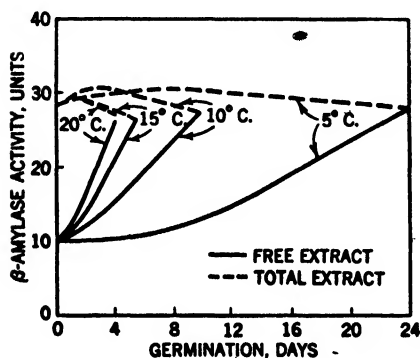


Fig. 1.—Free and total  $\beta$ -amylase activities in extracts of hard winter wheat germinated at different temperatures (130).

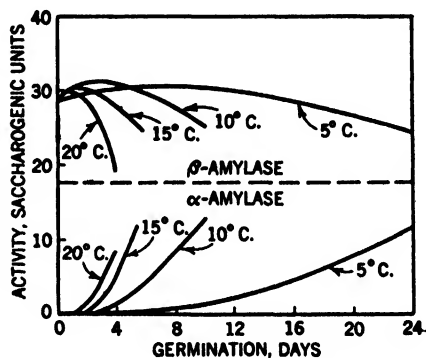


Fig. 2.—Changes in contents of total  $\beta$ - and  $\alpha$ -amylases of hard red winter wheat germinated at different temperatures (130).

amylase. Snider (217) has pointed out that cysteine should be employed in the papain digestion since this amino acid is an activator of papain and its use greatly reduces the digestion time required.

The studies already reviewed were conducted, for the most part, with barley. In 1942, Kneen, Miller, and Sandstedt (130) applied the techniques for determining  $\alpha$ - and  $\beta$ -amylase activity developed in their laboratory to a study of the changes in amylase activity which occurred during the germination of hard red winter wheat at different temperatures in the laboratory. The ungerminated wheat contained relatively large amounts of total  $\beta$ -amylase (Fig. 1), but the  $\alpha$ -amylase activity (Fig. 2) was extremely low. Approximately one-third of the  $\beta$ -amylase and one-half of the  $\alpha$ -amylase was free. With germination, the proportion of free  $\beta$ -amylase increased until it became equal to the total  $\beta$ -amylase. The total

$\alpha$ -amylase activity increased from 0.04 unit in the original wheat to 238 units after 5 days' germination (15° C.); most of the  $\alpha$ -amylase was in the free condition. Calculations based on the dry weight showed that there was an apparent increase in the amount of total  $\beta$ -amylase during the initial stages of germination, followed by a pronounced and progressive decrease; the free and total  $\alpha$ -amylase activity, however, progressively increased throughout the germination period. These studies were not conducted under malting conditions, but Geddes, Dickson, and Croston (75) have recently analyzed composite samples of malts representing four market classes of American wheats germinated under malting conditions.

The data are summarized in Table I, and confirm the general trends noted by Kneen *et al.* (130). It thus appears that  $\beta$ -amylase is activated or released upon germination, whereas there is an actual synthesis of  $\alpha$ -amylase.

TABLE I  
EFFECT OF GERMINATION ON FREE AND TOTAL  $\alpha$ - AND  $\beta$ -AMYLASES OF WHEAT\*

Germination moisture, per cent	Time, days	$\alpha$ -Amylase			$\beta$ -Amylase		
		Free	Total	Free as % total	Free	Total	Free as % total
35	0	..	..	...	3.8	20.0	19
	2	3.8	4.6	83	4.5	19.6	23
	3	9.3	10.5	88	4.9	19.7	25
	4	17.9	21.2	84	5.4	20.3	27
	5	28.8	30.8	93	6.6	20.4	32
	6	43.8	42.0	104	7.6	20.6	37
40	2	13.6	15.3	89	4.6	20.1	23
	3	36.6	36.7	100	6.0	20.6	29
	4	58.6	58.6	100	7.8	20.5	38
	5	68.5	71.9	95	9.1	20.5	44
	6	89.6	91.0	98	10.1	20.5	49
45	2	19.0	19.0	100	6.7	19.8	34
	3	39.8	39.6	100	8.3	20.2	41
	4	61.4	65.2	94	9.5	20.4	47
	5	76.1	75.3	101	10.2	20.4	50
	6	91.4	96.1	95	10.3	21.0	49

\* Unpublished data of Geddes, Dickson, and Croston (75) obtained with composite samples of malts, experimentally produced at 16° C. from representative samples of soft white, soft red winter, hard red spring, and hard red winter wheats. Free and total  $\alpha$ - and  $\beta$ -amylase were determined by the methods employed in a similar study by Kneen, Miller, and Sandstedt (130). The  $\alpha$ -amylase activity unit is the number of grams of soluble starch which is dextrinized by the  $\alpha$ -amylase of 1.0 g. malt in 1.0 hr. at 30° C., in the presence of an excess of  $\beta$ -amylase (201). The  $\beta$ -amylase unit is the number of grams of soluble starch converted to maltose by the  $\beta$ -amylase of 1.0 g. malt in 1.0 hr. at 30° C. (131).

In a comparative study of the development of  $\alpha$ - and  $\beta$ -amylase activity during the germination of single samples of different cereals, Kneen (125)

found that barley, on the average, contains more  $\beta$ -amylase than wheat, but the production of  $\alpha$ -amylase is greater during the germination of wheat. All the ungerminated cereals evidenced low, but measurable,  $\alpha$ -amylase activity. The properties of the wheat amylases appear to correspond rather closely to those of barley (93-95).

The amylase activity of different wheats malted under uniform conditions varies widely. Thus, Dickson and Geddes (59) found the amylase activities of malts prepared from average samples of soft white, soft red, hard red spring, and hard red winter wheats, grown in the United States, to decrease in ~~the~~ order named. Meredith, Eva, and Anderson (166) found that the amylase activities of malted wheat flours prepared from hard red spring wheat malts exhibited marked differences due both to the variety of wheat and points of growth. The  $\alpha$ - and  $\beta$ -amylase activities of the various malted wheat flours exhibited a low, but significant, association ( $r = 0.49$ ). However, the  $\beta$ -saccharogenic activity of the malted wheat flours was highly correlated ( $r = 0.90$ ) with the total saccharogenic activities (papain extracts) of the unmalted flours.

## 2. *Variations in Amylase Activity of Wheats and Flours*

As previously noted, sound wheats and the flours milled from them contain relatively large quantities of  $\beta$ -amylase (free and bound) and very small amounts of  $\alpha$ -amylase. Upon germination, the free  $\beta$ -amylase increases at the expense of that which is latent and there is a marked production of  $\alpha$ -amylase. Elizarova (62) has reported that spring wheats average higher in  $\beta$ -amylase activity than winter wheats. Kneen and Hads (129) noted variations in the  $\beta$ -amylase activity of hard red winter wheats according to variety and place of growth; within varieties, the total  $\beta$ -amylase was positively correlated with protein content.

There are many reports in the literature in which the saccharogenic activities of extracts of various flours, acting upon a common substrate, have been found to be essentially similar. On the other hand, Dadswell and Wragge (53) found variations of from 7.9 to 106.5 g. in the maltose produced by water extracts of Australian bread flours (milled from different varieties grown at several stations) acting upon soluble starch. In a study of 23 Canadian spring wheat flours, experimentally milled from different varieties of hard red spring wheat, Meredith, Eva, and Anderson (166) found the total saccharogenic activity (papain extracts) to vary from 26.8 to 42.1 units. Further studies are needed to determine the variations in activity which exist among different market classes, grades, and varieties of wheat grown at different stations.

## V. Factors Affecting the Maltose Value of Wheat Flour

### 1. Introduction

It has long been recognized that autolytic determinations of the Rumsey type measure the summation of the effects of amylase concentration and starch susceptibility, and many studies have been conducted to elucidate the relative significance of these two variables. The maltose found after autolysis of a flour-water suspension has been shown to be influenced by a number of factors, including the class and variety of wheat from which the flour was made, the environment of the wheat during growth and harvesting, and the milling treatment which it receives.

### 2. *Amylase Content and Starch Susceptibility in Relation to Maltose Value*

The researches of early workers (36, 106, 191, 224, 237) which have been reviewed by Alsberg (1) clearly indicated that variations in the susceptibility of the starch to amylase attack would be expected to influence the autolytic production of maltose in wheat-flour suspensions. Alsberg, himself, recognized three factors: "diastase content," injured granule content, and the susceptibility of the uninjured granules.

That differences in the extent of mechanical injury of the starch granules are the principal cause of the variations in the maltose values of high-grade flours milled from sound wheats has been demonstrated by many investigators. Mangels (155) found that aqueous extracts of flours which differed appreciably in maltose figure gave very similar values when added to the same starch substrate; on the other hand, a given extract, acting on different starches, resulted in widely different maltose values. Hermano and Rask (95) and Malloch (153) also demonstrated differences in wheat starch susceptibility by the use of uniform amylase preparations. Later observations, which will be reviewed in a following section, have demonstrated that experimentally milled flour is lower in maltose value than commercially milled flour from the same wheat, and that overgrinding flour markedly increases the maltose values. Sandstedt, Blish, Mecham, and Bode (199) also demonstrated that the susceptibility of the raw uninjured starch in different samples of amylase-free flours to a given amylase extract is virtually the same, and that differences in maltose value exhibited by the inactivated flours are due to the varying quantities of susceptible starch which they contain. Andrews and Bailey (7) observed that the increase in rate of maltose production affected by the addition of uniform dosages of germinated wheat flour to suspensions of normal flours was positively correlated with the maltose-producing abilities of the normal flours. This



indicated that flour maltose values are more a function of the condition of the starch than of the actual content of amylases. This conclusion was substantiated by the fact that water extracts of several flours possessing widely different maltose values yielded nearly equal quantities of maltose when acting upon a soluble starch substrate. Sandstedt *et al.* (199) concluded that the higher maltose production obtained with commercially milled than with laboratory milled flours was due to the fact that the former contained larger proportions of the more susceptible starch fractions. Blish, Sandstedt, and Kneen (23) showed that autolytic maltose production is very rapid at first, but after an hour or more it diminishes to a very low rate, a fact which is attributable to exhaustion of the available starch substrate, and not to loss of active enzyme. They further showed that the small amount of the starch normally converted is substantially increased by additional grinding, and that a portion of the starch in flour which is susceptible to  $\beta$ -amylase attack is the result of mechanical injury during milling. They suggest, however, that a significant amount may occur naturally in the form of dextrins or closely related substances.

All flours contain an abundance of  $\beta$ -amylase (23, 220), so that the quantity of available or susceptible starch is the limiting factor governing autolytic maltose production. This view is supported by the observations of Dadswell and Wragge (53) who noted that while the amylase activities of the water extracts of a series of Australian flours varied widely, the activities were not related to the maltose value of the flours.

Sandstedt, Jolitz, and Blish (200) produced additional evidence in support of the concept that the quantity of susceptible starch is the important variable in autolytic maltose production by washing the gluten from flour-water dough and recovering the starch from the wash water by centrifugation. As is well known, two distinct layers of material are obtained. These authors observed that the depth of the upper layer varied for different flours, increasing in this order: soft wheat flour, experimentally milled bread wheat patent flour, commercially milled bread wheat flour, and durum flour. While the lower layer was relatively pure starch, the upper layer appeared to be impure highly hydrated dextrins which resulted from the action of the flour amylases on the "available" starch and was tentatively designated as amyloextrin. In a more detailed study of the so-called amyloextrin fraction, MacMasters and Hilbert (151) found that it is composed of the very small starch granules, varying amounts of damaged large granules (depending upon the severity of milling) which gelatinize in cold water, smaller quantities of protein, fatty acids, ash, and pentosans together with cellulose in the cell wall debris.

The visible detection of starch granules which are susceptible to amylase attack was carried out by Brown and Heron (36) in 1879 and more recently by Huss (107), Pulkki (187), Dadswell and Wragge (53), and Jones (111).

Huss observed that uninjured raw starch granules were not stained by eosin, Congo red, or iodine, but when injured mechanically or heated in water to the initial swelling stage, they took up the stain.

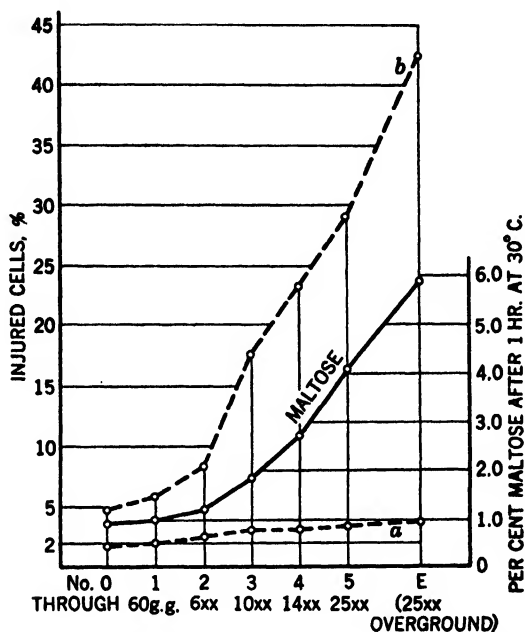


Fig. 3.—Relation of maltose value to different types of injured starch granules in wheat flour as influenced by fineness of grinding (187). Curve *a* represents the percentage of starch granules of  $20\ \mu$  diameter or over which are broken; curve *b* represents the percentage of starch granules of  $20\ \mu$  diameter or over which stain with Congo red.

Pulkki (187) showed that it is possible to differentiate between two groups of wheat starch granules by the simultaneous use of iodine and Congo red:

(a) *Grains which were visibly fractured to a greater or lesser degree.* Most of these stained completely red although some were found which only stained red in the region of the fracture, while the rest of the grain stained blue. In this group there were also a few grains which stained entirely blue.

(b) *Grains which stained either entirely or partly red.* In most of these, no visible fracture could be detected. The number of broken granules (group *a*) was augmented very little by overgrinding flour, but the maltose value

ultimately increased sixfold. In contrast, as shown in Figure 3, the number of red-stained granules which were seemingly uninjured (group b) increased very rapidly with overgrinding and paralleled the increases in maltose value. It is significant that, as a rule, only the large granules stained with Congo red; granules less than  $20\ \mu$  in diameter stained entirely blue and appeared perfectly intact.

In an attempt to explain these observations, Pulkki suggested that the starch granules of wheat are surrounded by a layer or film which, unlike the inside material of the granule, is not readily attacked by amylase nor stainable with Congo red and can easily be removed by mechanical means. The photomicrographic studies of Sandstedt (196), however, have shown that differences in the resistance of wheat starch to amylase action are not due to a granule membrane, but are associated with granule structure. Pulkki concluded that if some entirely different method of grinding (other than rolls) were used, "it would be at least theoretically possible that a high degree of fineness could be reached without effecting any great changes in the amylolytic activity of the material."

In a study of Australian flours, employing a slight modification of the staining technique used by Pulkki (187), Dadswell and Wragge (53) also found a high correlation between the

percentage of the granules over  $20\ \mu$  in diameter which stained with Congo red and the maltose value. Their data, together with the results given by Pulkki which are directly comparable, are shown graphically in Figure 4. The total correlation between the percentage of starch granules which stained with Congo red and the maltose value based on four varieties grown at six stations was  $+0.93$ ; when the effects of variety and station were removed, the correlation became  $+0.70$ . When two commercial flours were overground, the maltose value was increased 2.1 times and the percentage of starch stainable with Congo red increased by an average of

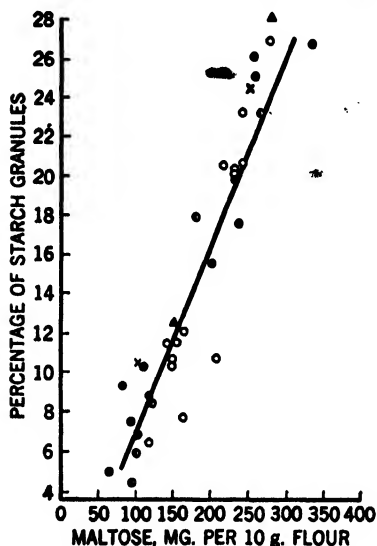


Fig. 4.—Relation between maltose formed upon autolysis of wheat flours for one hour at  $30^{\circ}\text{C}$ . and percentage of starch granules of  $20\ \mu$  diameter or over which are stained with Congo red (53).

2.3 times. Yet the maltose produced by the enzymes extracted from the original and overground flours acting on a common substrate was essentially equal. Sedimentation tests showed that flours which suffered extensive damage to their starch settled out of a suspension more slowly than flours which exhibited little damage to the granules; this is associated with the swelling of the injured granules.

In an extensive microscopic study of the production of damaged starch in the milling of wheat in relation to autolytic maltose production, Jones (111) recognized two types of mechanical action on the starch which he designated as the "surface factor" and the "internal factor." The "surface factor" is due to the shearing or scraping of material from particle surfaces and is influenced by the nature of the roll surface and the differential in roll speed. The internal factor is due to the crushing or partial flattening of the larger particles, and the extent of the damage depends upon the size and hardness of the particles and on the roll pressure. Both factors produced the same type of damage. Instead of an expected increase in the number of cracked or split granules as a result of mechanical action, Jones observed that the damage was characterized by an increase in the number of granules which had a curious flat appearance and a thin faint outline which suggested their designation as "ghosts." The larger granules (diam. 30-35  $\mu$ ) were the ones which were principally involved in this phenomenon. While 0.3% iodine solution stained all starch granules indiscriminately, on irrigating a flour-water slide with the iodine solution, the ghosts stained more rapidly and, at first, more deeply than the sound granules. If 0.02% iodine solution was used, the ghosts were preferentially stained and the color could be leached from them by irrigation with water. This is illustrated in Figure 5. Similarly, Congo red solution (0.1% to 0.35%) stained the ghosts a uniform orange-pink color, but did not stain sound starch. Microscopic observations indicated that the ghosts are produced by a complete peripheral rupture of the lenticular granule with the result that it divides into two superimposed portions. This cleavage permits the ready attack of these characteristically injured granules by amylase, as shown by the complete disappearance of the ghosts—and only the ghosts—upon treatment with a flour amylase preparation. Jones demonstrated that amylase is uniformly distributed throughout the endosperm of sound wheat and that the granularity, or particle size, of flour and milling stocks is not in itself a factor determining the maltose value. In flours and intermediate stocks, milled to varying degrees from a given wheat, the maltose figure is a measure of the number of ghosts present.

Alsberg and Griffing (2) observed that overgrinding flour injures the

starch granules so that part of the starch swells and disperses when water is added to form a dough. Stamberg and Bailey (222) demonstrated that raw wheat starch which had been finely pulverized by grinding in a rod mill for 48 hours was about as easily hydrolyzed by  $\alpha$ - and  $\beta$ -amylases as was

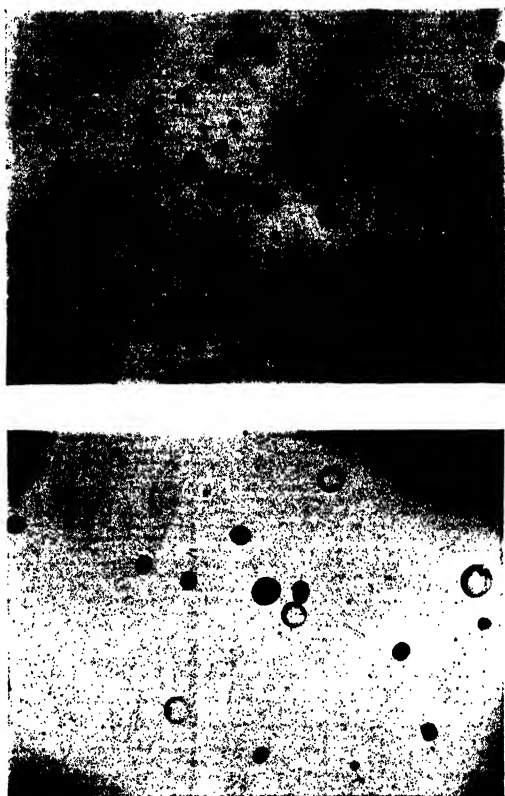


Fig. 5.—Photomicrographs (111) showing indiscriminate staining of all starch cells with 0.3% iodine solution (upper plate) in contrast with preferential staining of the "ghosts" with 0.02% iodine solution (lower plate).

soluble starch. In this connection, it is of interest to note the observation of Lampitt, Fuller, and Goldberg (134) that the proportion of wheat starch extractable by cold water increases upon dry grinding in a ball mill until, after grinding for 2000 hours, nearly all the starch becomes soluble.

Wheat starch is composed of granules which differ markedly in size and Whympster (237) reached the conclusion that the large granules are more

readily attacked by amylase than the small granules. Grewe and Bailey (83) measured the proportion of small starch granules (less than  $7.4\ \mu$ ), intermediate granules ( $7.4$  to  $14.8\ \mu$ ), and large granules (over  $14.8\ \mu$ ) in starches prepared from seventeen flours milled from various classes of wheat in the United States. Employing their data, Stamberg (219) calculated the size distributions according to number, weight, and surface area, using the value of 1.5 as the density of starch and assuming the granules to be perfect spheres. The average distributions on these three bases are given in the table below.

SIZE DISTRIBUTION OF STARCH GRANULES IN PER CENT

Description	Small granules ( $<7.4\ \mu$ )	Intermediate granules ( $7.4$ – $14.8\ \mu$ )	Large granules ( $>14.8\ \mu$ )
Number	81.6	5.9	12.5
Weight	4.1	2.9	93.0
Surface area	17.7	5.9	76.4

The average surface area per gram of wheat starch was 2004 sq. cm., and the individual flours showed variations in surface area of from 88.8 to 116.7% of the average. The total surface area was, of course, closely correlated with the number of small starch granules and using this criterion, Grewe and Bailey (83) were unable to discern any correlation between granule size and the source or type of wheat, or the maltose values of the flours. More recently, Stamberg and Bailey (222) separated wheat starch into small (diam.  $3$ – $6\ \mu$ ) and large granules (diam.  $20$ – $35\ \mu$ ) and tested their susceptibility to saccharogenesis by  $\alpha$ -amylase. The small granules were found to be slightly less resistant than the large granules, but these differences were quite insufficient to account for the observed differences in the amylolytic susceptibility of the various raw starches they studied. Neither the small nor large granule fractions were as readily hydrolyzed as the original starches from which they were prepared, which suggests that the additional washing required to fractionate the granules had removed the more labile material.

Although it is clear that variations in the quantities of mechanically injured starch granules are chiefly responsible for the differences which are observed in the autolytic production of maltose by wheat flour suspensions, the question arises whether any inherent differences exist in the susceptibility of starches from different varieties and classes of wheat. In an endeavor to answer this question, Stamberg and Bailey (222) allowed an active  $\alpha$ -amylase preparation to act on different raw starches for 24 hours,

when a second addition of  $\alpha$ -amylase was made. The same relative susceptibility of the raw starches was observed in each case, and it was concluded that differences do exist in the susceptibility of raw starches. Reitz (192) isolated starches from flours experimentally milled from different genetic species of wheat. The starches were purified by repeated washings with distilled water. Approximately 140 gallons of water were used in the preparation of each starch and the upper layer of starch, comprising the so-called amyloextrin fraction of Sandstedt *et al.* (200), was repeatedly discarded. No injured granules were observed in the final preparations, and it was believed that they were removed by the prolonged washing to which the starches were subjected. Representative maltose values for starches from the different species are given in the table below.

Wheat	<i>Triticum</i> species	Maltose units, mg.
Red winter spelt	<i>spelta</i>	14
Einkorn	<i>monococcum</i>	21
Little club	<i>compactum</i>	23
Thatcher	<i>vulgare</i>	52
Yaroslov Emmer (North Dakota)	<i>dicoccum</i>	80
Polish (Washington)	<i>polonicum</i>	101
Mindum	<i>durum</i>	115
Poulard (Washington)	<i>turgidum</i>	194
Khapli Emmer (Washington)	<i>dicoccum</i>	204

These results are indicative of inherent differences in starch susceptibility. That uninjured wheat starch is slowly attacked by  $\alpha$ -amylase has been observed microscopically by Sandstedt (196).

### 3. *Effect of Wheat Variety and Environment on Maltose Value*

The maltose found after autolysis of a flour-water suspension has been shown to be influenced by the class and variety of wheat from which the flour was made (47, 53, 142, 143, 156, 162, 226, 236) and the environment of the wheat during growth and harvesting (10, 32-34, 53, 64, 96, 110, 119, 142, 155, 157, 162, 227).

Maltose values determined for various market classes of sound wheats and for the flours milled from them are related to the hardness or vitreousness of the wheat; durum wheat yields the highest values, followed by hard red spring, hard red winter, and soft red winter in the order named. Hard types of white wheats give higher maltose values than the soft types. In addition, maltose values for different varieties within any one class of wheat have been found to vary rather widely. Thus, Mangels (155) secured values ranging from 66.5 to 134.6 among straight grade flours milled from

different varieties of hard red spring wheat grown at the same station. Similarly, Markley and Bailey (162) reported rather wide varietal, as well as station, differences. In many instances, the varietal differences in maltose value appear to be associated with the hardness of the kernels, the more vitreous varieties exhibiting the higher values.

It is well known in the grain trade and verified by many laboratory studies that drought conditions which are not sufficiently severe to result in improper filling of the kernel yield wheats and flours of low maltose value. When locations of growth are arranged in order of increasing rainfall during the growing season, the wheats (or flours) from these stations are, in general, arranged in order of increasing maltose value. Flours milled from wheats grown under irrigation average appreciably higher in maltose value than those grown under dry-land conditions. Mangels (155) has reported that cropping systems and fertilizer treatment influence maltose value; the application of manure and phosphatic fertilizer caused an increase in maltose value, whereas potash fertilizer had a depressing effect.

Mangels and Stoa (157) and Malloch (153) have shown that maltose value tends to decrease as the wheat matures. Bracken and Bailey (32) found that delayed harvesting after the grain is ripe was without any significant effect on the maltose value of hard winter wheat. This observation was confirmed by Swanson (227) who showed that alternate wetting and drying of wheat, such as may occur during harvesting, does not influence maltose value unless the process of germination actually begins. Sprouting of wheat, however, markedly increased maltose value, the increase being greater in the germ than in the brush end of the kernel. In a study by Malloch (153), flour experimentally milled from a sample of unsprouted wheat gave a maltose value of 146 units, as compared with 834 units for flour milled from badly sprouted wheat (sprouts 0.75–1.5 in. long).

Flours milled from wheat which has been frosted before it has reached full maturity have higher maltose values than those milled from normal wheats.

#### *4. Effect of Milling Treatment on Maltose Value*

Several variables in the milling process itself, such as the tempering treatment, the relative humidity of the mill atmosphere, the manner in which the stocks are classified, the fineness of grinding, the differential in roll speed, and the pressure exerted during grinding have been found to influence the maltose value of wheat flour (74, 84, 96, 143, 162, 213, 220, 226, 242).

In an experimental study of certain milling factors, Markley and Bailey (162) found that increasing the moisture content at which the wheat was



milled, as well as increasing the relative humidity of the mill room, lowered the maltose value of the flours; on the other hand, increasing the fineness of the flour particles markedly increased the maltose value. It is significant that the effect of fine grinding was greater when the milling was carried out at a low relative humidity. Thus, wheat samples ground at 50% relative humidity to pass a 10XX silk sieve gave a mean maltose value of 391 units, as compared with 556 units when ground to pass a finer sieve (16XX). When milled at 75% humidity, the corresponding values for flours ground to pass the coarse and fine sieves were 344 and 376 units, respectively. The authors concluded that particle size *in itself* is not a major factor in determining the maltose value of a flour.

Previously, many workers had shown that the more finely granulated material in a flour sample was higher in maltose value than the more coarsely granulated fractions. It is now clear that the higher maltose values of such flours is associated with greater mechanical injury to the starch. It was shown by Brown and Heron (36) in 1879 and by Maquenne (158) in 1904 that mechanically injured starch granules are the more readily attacked by amylase. In 1925, Alsberg and Griffing (2) showed that the enzymic hydrolysis of starch was increased by the fine grinding of flour, and this observation was confirmed and extended by Pascoe, Gortner, and Sherwood (184), Leatherock, McGhee, and Giertz (143), Dadswell and Wragge (53), Ziegler (242), and many other workers. Thus, Pascoe *et al.* increased the maltose value of a commercially milled flour approximately 35% by regrinding it in a ball mill for 24 hours. Several investigators (143, 162, 163, 184, 199, 226) have shown that flours milled on a small experimental mill are appreciably lower (34–68%) in maltose value than flours commercially milled from the same wheat. This is associated with the lesser degree of mechanical injury in experimental milling.

Ziegler (242) has investigated the effect on flour maltose value of the nature of the surface, the pressure, the differential, and the temperature of the reduction rolls in milling. When a differential in the speed of the rolls existed, the rougher the roll surface and the higher the roll pressure, the greater was the increase in flour maltose value. Smooth rolls developed more heat in milling than frosted or scratched rolls and as the roll temperatures increased, there was a marked increase in the maltose value of the flour; from 30° to 80° C., the increase was approximately linear. In the absence of a speed differential, variations in the surface (polished, frosted, or scratched) of the rolls or the roll pressure were without any significant influence on the flour maltose value. Similarly with a polished roll surface, variations in speed differential or roll pressures had no appreciable effect on

the maltose value. Ziegler concluded that the higher maltose values of commercially milled flours, as compared with experimentally milled flours, are doubtless in part the result of the higher roll temperatures involved in the former process.

Jones (111) differentiated between two types of starch injury during milling: (1) the "surface factor," due to the shearing of material from the particle surfaces, which is influenced by the roll surface and the speed differential; and (2) the "internal factor," due to the crushing or partial flattening of the larger particles, which depends on the size and hardness of the particles and on the roll pressure. Reference was made on page 433 to his observation that mechanical injury in milling produces a type of starch damage which is characterized by the presence of starch granules, designated as "ghosts," which have a curious flat appearance and thin faint outline and which stain completely with iodine and Congo red.

Although the ghosts are produced by both the shearing and crushing action encountered in roller milling, the distinction between the two factors is important in determining the maltose value of the final straight grade flour. In this connection, Jones has pointed out that with stocks of different particle size coming directly from the break rolls, the maltose value remains low as the particles become finer. However, the operation of the internal factor causes the coarser fractions from the reduction rolls to be relatively high in maltose value because the particles have "internal" zones of damaged starch as a result of the strain caused by the partial flattening of the particles in passing between the rolls. This strain is much less pronounced with particles of intermediate size. Accordingly, when a coarse primary stock is passed once through the smooth reduction rolls, the maltose values of the coarser fractions of the grind are relatively high, while those of the intermediate fractions are low. The coarser the particles and the heavier the roll pressure, the higher will be the resulting maltose value. The flour produced in grinding such coarse particles has a relatively high maltose value which is markedly influenced by the nature of the roll surface and the differential in roll speed, that is, by the surface factor.

Incompletely reduced particles carry damaged starch which becomes liberated, in addition to that freshly produced, when they are further reduced. This is the cause of the high maltose figures for flour and other stocks from later reductions, the magnitude of the values depending largely on the conditions of the earlier reductions. That is, the effect of the internal factor is cumulative, the maltose values of the coarser particles increasing markedly after each reduction. With gentle reduction of fine stock, few ghosts are produced, but an increase in the roll pressure markedly increases

the maltose value of the flour and, more particularly, of the remainder of the grind. Jones has also pointed out that the extent to which the internal factor is operative is greater the harder the wheat, particularly under severe milling conditions. Consequently, the differences in the maltose values of flours milled from different types of wheat are to be attributed, in part at least, to the effect of differences in the hardness of the endosperm on the extent of the damage to starch during milling.

The relative maltose values of the various flour streams which are obtained in the commercial milling of wheat remain to be considered. Pascoe *et al.* (184) found a difference of 169 units between the streams of the highest and lowest maltose value. In analyses by Leatherock *et al.* (143) of the flour streams of an 1800 barrel mill operating on hard red winter wheat, the maltose values of the flour streams varied from 132 to 488 units. The streams could be roughly classified into three groups in order of increasing maltose value: group I, break flours (208–245 units); group II, middlings flours (269–296 units); and group III, low-grade flour streams (277–488 units). The highest maltose values were obtained for the first sizings flour, which is rich in germ. In general, an increase in maltose values was associated with an increase in the ether extract and ash content of the flour. Harris and White (91) obtained very similar results in a study of mill streams obtained in the commercial milling of hard red spring wheat.

Summarizing the effects of wheat variety, of environment during growth and harvesting, and of milling treatment, it seems apparent that variations in amylase content and in starch susceptibility may both contribute to the differences which occur in flour maltose values. An increase in  $\alpha$ -amylase content is doubtless the main factor responsible for the high maltose values of flours milled from immature, frosted, and sprouted wheats, and also probably contributes to the high values observed in low-grade flours which are contaminated with germ. On the other hand, the extent of mechanical injury of the starch, which increases its susceptibility to attack by the amylases, is undoubtedly the principal factor involved in variations in the maltose values of flours milled from sound wheats. Thus, the softer the wheat, the higher the moisture content at which it is milled, the less the differential in speed of the reduction rolls, the lower the roll temperature, the smoother the roll surface, the lower will be the maltose values of the flour. These are all conditions which may be expected to result in less mechanical injury to the starch. It is significant to recall that the break flours which are released early in the milling process are considerably lower in maltose value than the middlings and other flours which have been subjected to the action of the reduction rolls.

In America, the bread wheats are of harder types than European wheats, and they are milled at lower moistures after shorter conditioning periods and at higher roll pressures and roll speeds than are prevalent in Europe. Greater mechanical injury to the starch would, therefore, be expected under American milling conditions. In this connection, Kosmin (123) has shown that the European wheat-conditioning systems lead to the production of flour of low maltose value.

## VI. Relation between Autolytic Maltose Production and Flour Gassing Power

Baking technologists have long recognized the importance of adequate gas production in breadmaking, and the primary purpose in determining flour maltose values (autolytic production of maltose in flour-water suspensions) is to evaluate the capacity of different flours to support yeast fermentation. Some, however, prefer to obtain a measure of this flour characteristic directly by measuring the gas produced by a flour-water-yeast dough in a given time; that is the *gassing power* of the flour. While it was early established that a positive correlation existed between these two measures, there has been some difference of opinion as to whether or not they can be used interchangeably for control purposes. Jørgensen (113) considered that, for practical purposes, flour maltose value and flour-gassing power may be regarded as synonymous terms and this view is supported by the close relation found between these variables by several workers (57, 81, 142). On the other hand, Karácsonyi and Bailey (116) found that overgrinding wheat flours resulted in substantial increases in flour maltose value but had little influence on the gassing power. The sucrose content of wheat flours varies considerably and as sucrose is not measured in the determination of flour maltose value but supports yeast fermentation, this influences the relation between these variables (22). Moreover, the autolytic production of maltose is measured for one hour at constant pH with accumulation of end products, whereas in actual gas production tests the rate of amylase action increases with time as a result of the increase in dough acidity and the removal of end products by fermentation. Under these conditions, an exact parallelism between flour maltose value and gas production could not be expected to exist (137).

Eva, Geddes, and Frisell (63), in a study of 49 wheat flours, varying in maltose value from 99 to 278 units, obtained a significant correlation of  $+0.79$  between maltose value and six-hour gas production. This correlation is not sufficiently high to permit accurate prediction of gas production from flour maltose value. Subsequently, Fisher, Halton, and Hines (68)

surprisingly found no correlation between the maltose figure and gas production, either during the final proof or throughout the whole course of fermentation, and conclude that the routine maltose test cannot be safely used as a substitute for the more tedious and cumbersome gassing test. Bottomley (27) also found a rather low relationship between these variables and suggested that the main use of maltose value determinations is in mill control work where the type of flour under consideration varies only within narrow limits.

The finding of Hildebrand and Geddes (98) that a curvilinear relation (Fig. 6) existed between gas production and maltose value for a series of

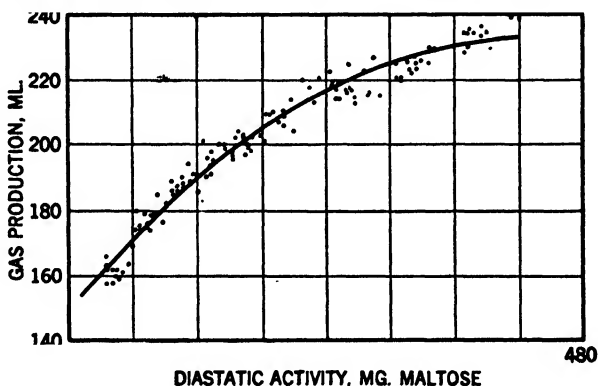


Fig. 6.—Relation between maltose value and gas production for flour blends made by adding increments of different malted wheat flours to a common base flour (98).

flour blends made by adding small increments of malted wheat flours of varying saccharogenic activity to a common base flour is of interest. Here, the substrate was essentially uniform as regards sucrose content and starch susceptibility, and under these conditions a high correlation existed ( $r=0.98$ , based on a nonlinear regression). Subsequent tests by the writer have shown that the relation could not be rendered more linear by increasing the percentage of yeast in the gassing power determination, so that the curvilinearity cannot be explained as being due to the production of sugar at a faster rate than it is being metabolized by the yeast.

It seems probable that the relation between these variables might be improved by carrying out the autolysis for a longer time. Sandstedt *et al.* (199) have shown that the rate of autodigestion of different flours falls off as the time is extended. The decrease in the rate varies for different flours due, presumably, to variations in the quantities of susceptible starch which

are present. By extending the time of autolysis, a better differentiation between flours would presumably be obtained and the quantity of sugar produced might more closely approach that of the fermentation test. Such a modification of the maltose value test would, however, eliminate the advantage of the rapidity with which it can now be carried out.

The researches summarized here show that the gassing power test is the more informative and although more time consuming and cumbersome, it is, to a large extent, replacing the maltose value determination. This is indicated by the large number of devices, some of which are automatic, which have been developed in recent years (see Section III, page 424) to simplify the measurement of gas production.

## VII. Biochemistry of Breadmaking

### 1. *General Survey of the Breadmaking Process*

It is beyond the scope of this chapter to deal with the various kinds of bread or the mechanical details of the process. For information on these topics, treatises on baking or the recent articles by Cathcart (43) may be consulted. Bread is made by two general processes—the straight dough, and the sponge and dough process. In the straight dough method, all the dough ingredients are made into a dough at one time; in the sponge and dough method, a portion of the flour and water, together with yeast, flour improvers (and sometimes the shortening) are mixed into a sponge of medium consistency; after a period of fermentation, the sponge is returned to the mixer where the remainder of the flour and water, and the salt, sugar, shortening, and milk solids are incorporated to form the dough. The formulas vary widely and in several European countries, bread is commonly made with only flour, yeast, salt, and water. The essential operations include mixing, fermentation, dividing or scaling, panning, proofing (fermentation in the pan), and baking.

In making a dough, sufficient water, including other liquid ingredients, is added to produce a dough of the proper consistency to pass through the dividing, rounding, and moulding machines with a minimum of tearing or sticking, and to yield an approximately optimum baked loaf from the particular flour. The quantity of water employed (which includes that present in the liquid ingredients) is designated as the *absorption* and is expressed as a percentage of the flour. During fermentation, some doughs increase in mobility or “slacken,” whereas others decrease in mobility or “tighten,” so that the consistency of the dough as it comes from the mixer must be adjusted to take account of these changes.

When water is brought into contact with the flour and other ingredients of the dough, a plastic, dynamic system of complex character is evolved. The flour proteins imbibe water to form gluten in which the starch grains are embedded. The yeast is dispersed in an aqueous medium which contains fermentable sugars, soluble nitrogen compounds, various inorganic salts, and vitamins which are essential for its metabolism. The carbon dioxide initially produced dissolves in the aqueous medium of the dough, thereby causing an increase in acidity; later, vesicles of carbon dioxide appear which mechanically expand the dough. The increase in acidity not only alters the physical properties of the gluten, but accelerates amylase activity. The amylases convert susceptible starch to dextrins and maltose and the increase in amylase activity as the fermentation progresses is an important factor in maintaining gas production during the later stages.

During the fermentation, marked changes occur in the physical properties of the gluten, as reflected by a decrease in the extensibility of the dough and an increase in its resistance to extension. Much of the art of baking is concerned with judging when this modification has reached the point which will result in an optimum loaf. When the dough is judged to be "ripe," it is subjected to the necessary processes involved in placing it in the pans where it undergoes further fermentation known as *proofing*.

After the dough has been proofed to the desired extent, it is placed in the oven where a sequence of physical and biochemical changes occur. As the temperature rises, the activity of the various enzymes is at first greatly accelerated and as the temperature continues to rise, the enzymes become inactivated. The gas produced by the accelerated fermentation, together with that formed by the distillation of carbon dioxide from the dough solution, undergoes thermal expansion, and greatly increases the volume of the dough until the coagulation of the proteins prevents a further increase in volume. To secure maximum volume, crust formation must be delayed by providing a relatively humid atmosphere in the oven. As the proteins become denatured by heat coagulation, their water-imbibing capacity is greatly reduced; on the other hand, the starch swells and undergoes a gelatinization which is limited by the amount of available water. Crust formation eventually ensues. This involves a desiccation of the surface of the dough, accompanied by caramelization of the sugars present.

## 2. Significance of Gas Production in Breadmaking

The success of the conversion of wheat flour into superior yeast-leavened bread depends largely on the gas-producing and gas-retaining capacity of the dough. Gas production depends upon the enzymic activity and the

nature of the carbohydrates in the dough, while gas retention is a function of the quantity and the colloidal characteristics of the gluten proteins. If gas production is not maintained at a sufficiently high rate, especially during the proofing period and the first few minutes in the oven, there will be insufficient gas to distend the dough and a small loaf of poor grain and texture will result. Such a loaf possesses a flat taste and pale crust color because of the low residual sugar content. A poor loaf will also be obtained if the dough does not retain a sufficiently high proportion of the gas which is produced. Thus, either gas production or gas retention may be a limiting factor in breadmaking. In 1907, Humphries and Biffin (105) formulated the following definition of strength which became a classic in the cereal chemical literature: "A strong wheat is one which yields flour capable of making large well-piled loaves, the latter qualification thus excludes those wheats producing large loaves which do not rise satisfactorily." It was many years, however, before this concept was universally accepted and the gas-producing ability of a flour was definitely regarded as a factor entirely independent of strength. The early studies on the factors involved in gas production in doughs and the development of our present concepts of flour strength will be briefly traced.

As far back as 1886, Jago (108) studied the autolytic production of sugar in flour suspensions, the gassing power of doughs, and the disappearance of sugar during fermentation. He was aware that dough fermentation is in part supported by sugar produced as a result of amylase action on starch. That the sugar content of dough decreased with fermentation was also observed by Parenti (183) in 1903, but Wood (241), in 1907, and his contemporaries were the first to realize its full significance. Wood found that the sugar content of a yeast-free dough increased with time. With some exceptions, he observed that the size of the baked loaf was positively correlated with the potential gassing power of the flour and that flour of low gassing power would not yield a loaf of satisfactory volume unless sugar was added to the dough.

Liebig (145) found that sucrose pre-existed in flour, and he isolated maltose as a product of amylase activity. He realized that yeast activity may reduce the sugar content to values which might limit the rate of gas production during the proofing period. Baker and Hulton (15) demonstrated that maltose was produced by amylase action on starch and differentiated between the saccharogenic and liquefying components. They observed that the liquefying component was either absent from certain flours or was unable to act. Although gas production increased with the general quality of the loaf, flours of poor baking quality were encountered



which possessed higher gassing power than flours of high breadmaking value. As a consequence, they concluded that gas retention appeared to be a more important factor than gas production. Ford and Guthrie (69) reached a similar conclusion. The gas produced during the last stages of fermentation was regarded by Humphries and Simpson (106) as of greater significance than the total gas evolved during the entire fermentation. They found more sugar in a fermented dough (made without added sugar) than was originally present, and regarded "diastatic activity" as being responsible for maintaining a satisfactory sugar level throughout the fermentation period.

Alway and Hartzell (4) failed to find a consistent relation between baking strength and gassing power (as determined in a flour suspension containing 12.5% yeast). Rumsey (194) stated that "the flour showing the greater diastatic power should show the greater strength and consequently the greater baking value, providing the relative quality and quantity of the gluten are the same." The same year, Collatz (49) concluded that "in all cases, the use of malt extracts gave a superior loaf of bread in volume, grain, and texture, thus increasing the baking strength of the flour." However, the studies of Wood (241) in 1907 and many other workers (39, 40, 50, 161, 164, 181, 229, 234) clearly indicated that deficiencies in gas production could be easily controlled in the formula by the addition of various "diastatic" supplements. Moreover, additional studies showed that flour maltose value, or "gassing power," was frequently not correlated with loaf volume (25, 184).

During this period, evidence was accumulating that the quantity and the colloid characteristics of the flour proteins were the primary factors involved in flour strength. Many papers were published in which high positive correlations were found between protein content and loaf volume, particularly where the comparisons were made within wheats of the same market class, and when the baking tests were carried out under conditions in which gas production was not a limiting factor. Still higher correlations have recently been obtained for the relationship between protein content and loaf volume within varieties; under these circumstances varietal differences in protein quality are eliminated.

As a result of these various lines of investigation, it is now clearly recognized that flour gassing power is a factor which can be directly controlled by the miller and the baker, and hence should be regarded as independent of its strength. This point of view has been emphasized by Kent-Jones (117), Fisher and Halton (67), Jørgensen (113), Blish and Hughes (19), and others.

Today, flour strength is a term which is used to designate, in a general

way, the inherent capabilities of a flour to produce good bread under conditions of adequate gas production. As already indicated, flour strength is primarily a function of the quantity and colloidal characteristics of the flour proteins. A strong flour has high water absorption and produces doughs of satisfactory handling properties which will yield bread of good volume, break, shred, crumb grain, and crumb texture over a considerable range of mixing and fermentation conditions.

### 3. *Yeast Fermentation in Sponges and Doughs*

The strains of yeast, *Saccharomyces cerevisiae*, which have been selected and propagated for use in bread manufacture possess high fermenting power. That wheat flour sponges and doughs contain the nutrients essential for yeast metabolism is exemplified by the fact that the rate of alcoholic fermentation in bread dough is much higher than that which is normally obtained in synthetic solutions. Thus, in a comparison of the rates of fermentation in Hansen's yeast nutrient solution (112), Slator's solution (215, 216), and a flour-water dough, Simpson (214) found that the rate in the dough was about 75% higher than in Hansen's solution, the better of the two synthetic media. Recently, however, Atkin, Schultz, and Frey (9) have shown that a solution containing potassium, magnesium, phosphate and sulfate ions, thiamin, pyridoxine, nicotinic acid, a source of yeast-available nitrogen, sucrose, and a sodium citrate buffer (pH 5.5) is able to support a rate of fermentation equal to that of a flour suspension fortified with an excess of sugar and available nitrogen, the two factors which frequently become limiting as dough fermentation proceeds. Their investigations revealed that the failure of previous workers to attain maximal fermentation in synthetic solutions was due to the omission of thiamin.

It is now clear that two independent groups of enzymes are involved in fermentation: (1) the invertase, maltase, and zymase system of yeast and (2) the amylases of the flour and other dough ingredients. These enzymes function in a complex and changing environment, but the researches of many investigators have elucidated the principal factors which influence the rate of gas production in doughs.

When water is brought in contact with the flour, yeast, and other ingredients of the dough, enzymic action begins. Freshly made doughs vary in hydrogen-ion activity from approximately pH 5.4 to 6.2, the precise value depending upon the grade of flour and the nature of the other ingredients (13, 19, 66, 67, 135, 223). Thus, patent flour doughs have a lower hydrogen-ion activity than clear flours by about 0.3 pH unit, and nonfat milk solids increase the initial hydrogen-ion activity by from 0.35 to 0.5 pH

unit. As fermentation proceeds, the acidity increases at a fairly uniform rate and at the completion of fermentation, the increase in hydrogen-ion activity of doughs is in the order of 0.2 to 0.5 pH unit, depending upon the extent of fermentation and the nature and amount of the buffer substances which are present. Throughout the fermentation, therefore, the hydrogen-ion activity is normally within the rather broad optimal range of about pH 4.0 to 6.0 for yeast fermentation (66, 67, 144), although it is above the optimal pH for the activity of the amylases, particularly  $\beta$ -amylase.

The sources of fermentable sugars in the dough are: the sugars present in the flour, the sugar added as a dough ingredient, and the maltose which is gradually produced by the action of amylases on starch. Determination of the sugars present in flour is complicated because of the difficulty of securing complete extraction without the production of maltose from the available starch by amylase action. Blish, Sandstedt, and Astleford (22) met this problem by extracting the flour with a dilute sulfuric acid solution at ice-water temperatures. In bread flours milled from sound wheat, they found only very small and nearly constant quantities of reducing sugars (approx. 0.1–0.2%) and from 1.0 to 1.74% of sucrose, based on the determination of reducing sugars before and after inversion. According to Colin and Belval (48), however, only a small part of the increase in reducing sugars which result from the acid hydrolysis of a flour extract is due to sucrose, the major portion arises from levosin, first isolated from cereals by Tanret (230) and described as a white, amorphous, faintly sweet, easily hydrolyzable, nonfermentable carbohydrate composed chiefly of fructose with a small quantity of glucose. Colin and Belval found that a typical white flour contained 0.17% of hexoses, 0.22% sucrose, and 0.60% levosin. While levosin is practically unfermentable in pure solution (48, 85), it largely disappears from fermenting doughs (48, 79) and hence must be regarded as a fermentable sugar in breadmaking. In America, sucrose, in quantities as high as 5% of the flour, is used as an ingredient of ordinary bread doughs; malt syrup (the principal sugar of which is maltose, together with some glucose) is also frequently used as a source of sugar. In the manufacture of yeast-leavened sweet goods, the sugar content may be as high as 22% (flour basis). Nonfat milk solids, which contain lactose, are also used in breadmaking, but as this sugar is not fermentable by bakers' yeast, it does not contribute to the fermentation.

Glucose is fermented at a slightly faster rate than fructose (102, 103, 140, 193, 218). As sucrose is hydrolyzed by yeast much more rapidly than it is fermented (78), its rate of fermentation is equivalent to that of the corresponding quantity of an equimolecular mixture of glucose and

fructose. Bakers' yeast has a much longer induction period for maltose than for other sugars (21), although the induction period is considerably shortened in the presence of glucose and sucrose (144, 206). Lanning (139) and Larmour and Bergsteinsson (140) have shown that maltose is fermented in a dough after glucose, fructose, and sucrose. The relative induction periods for glucose, fructose, sucrose, and maltose, as well as the general characteristics of gas production rate curves for fermenting doughs, are illustrated in Figure 7 taken from Larmour and Bergsteinsson (140). In a freshly mixed sponge or dough, the rate of fermentation at first is slow, but there is a rapid acceleration in rate until a maximum is reached.

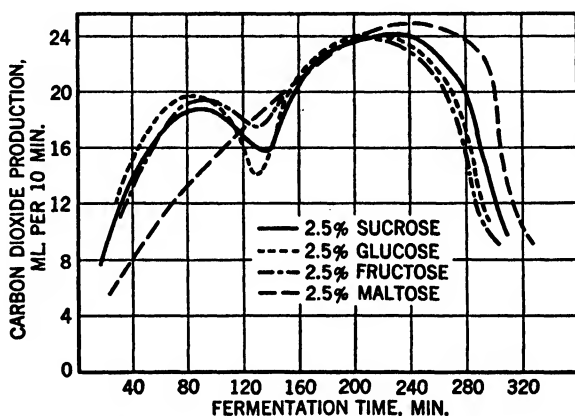


Fig. 7.—Rates of gas production by fermenting doughs made with sucrose, glucose, fructose, and maltose (140).

This maximum is essentially maintained, usually dropping slightly until the concentration of glucose and fructose (either present originally or arising from the hydrolysis of sucrose) is reduced to a limiting value, when a marked decrease in rate occurs. This phase is usually succeeded by an increase in rate to a second maxima (when the yeast commences the fermentation of maltose), followed by a second decrease in rate. During the first part of the fermentation, the rate of gas production in doughs made with flours of varying gassing power and with different amounts of added sugar is essentially the same if the percentage of yeast and the temperature are held constant (63, 78, 137, 140, 141). Later a differentiation in rate occurs, according to the sugar content of the dough.

The effect of increasing levels of sucrose on the rate curves for straight doughs containing 3% yeast is shown in Figure 8 from Larmour and

Bergsteinsson (140). As the percentage of sucrose is increased, the first maximum rate of fermentation is augmented and also shifted to a later period in the fermentation. The initial rapid rate of fermentation falls off when the sucrose percentage is reduced to a limiting value, and then rises to a second maxima when the fermentation of maltose begins. With 5% sucrose, the curve only shows one maximum, indicating that the sucrose is supporting the fermentation throughout the entire period. Landis and Frey (138) and Miller *et al.* (168) have also observed two maxima in the gas production rate curves for fermenting doughs.

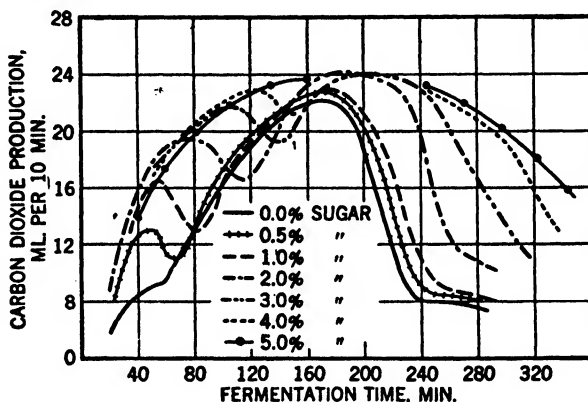


Fig. 8.—Rate of production of carbon dioxide by fermenting doughs made with varying amounts of sucrose (140). The quantities of sucrose are expressed as a percentage of the flour.

The influence of amylase action on the rate of gas production is well exemplified in Figure 9, taken also from Larmour and Bergsteinsson (140). The rate of gas production for three flours varying widely in maltose value is virtually identical for the first 100 minutes, during which the fermentation is supported by the sugars originally present in the flour and those added in the baking formula. It is only after the rate begins to decline from the first maxima that the variations in flour gassing power influence gas production. These data demonstrate the importance of amylase action in maintaining a high rate of fermentation during the later stages of fermentation.

The ultimate decline in gas production rate noted in Figures 7, 8, and 9 occurs in the presence of fermentable sugars, indicating that the fermentation is limited by a deficiency in other nutrients. Atkin, Schultz, and

Frey (9) point out that thiamin, pyridoxine, and nicotinic acid are present in white flour in adequate amounts, but it is not known whether all flours contain sufficient available magnesium, potassium, phosphate, and sulfate to promote prolonged dough fermentation. There is, however, ample evidence that the quantity of available nitrogen is frequently a limiting factor. Amos (5), Shen and Geddes (209), and Freilich and Frey (73) have shown that the amino nitrogen content of fermenting doughs markedly decreases with increasing fermentation time, due to the utilization of the soluble nitrogen constituents as food material by the actively fermenting yeast. Larmour and Bergsteinsson (140) observed that the addition of ammonium

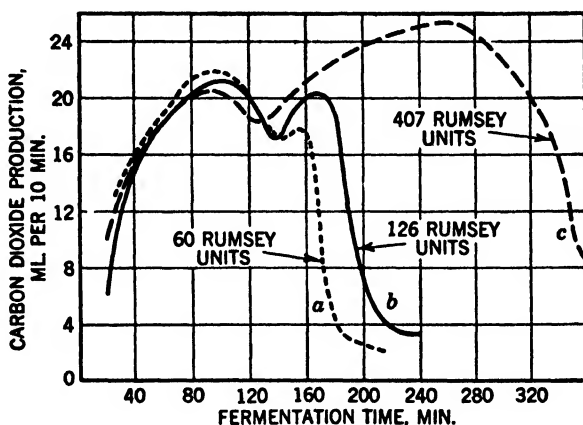


Fig. 9.—Rates of gas production by fermenting doughs made with flours of different maltose value (140).

salts to fermenting doughs increased the rate of gas production only during the later stages of fermentation (Fig. 10). Sandstedt and Blish (198) found that flours differed widely in the third-hour fermentation rate when tested in doughs containing 3% yeast and 4% maltose. The differences were ascribed to variations in the "factor M" content of the flours, a factor which was suggested as a specific catalyst for maltose fermentation. Schultz, Atkin, and Frey (207) showed that various amino acids significantly increased the third-hour fermentation rate and suggested that the differences noted by Sandstedt and Blish might be due to variations in the amino nitrogen content of the flours they examined. This suggestion was supported by the observation of Ofelt and Sandstedt (177) that the addition of ammonium salts and hydrolyzed gluten to doughs made from a number of flours resulted in a very uniform rate of third-hour gas produc-

tion. They also noted that the third-hour proof rate for a series of flours increased in nearly linear fashion with their protein content. These researches show that the quantity of available nitrogen is frequently a limiting factor in the later stages of dough fermentation. It seems probable that the beneficial effects of an increase in the  $\alpha$ -amylase activity of doughs on the rate of fermentation during the proof period (see page 454) may be due in part to an accompanying increase in proteolytic activity. For a

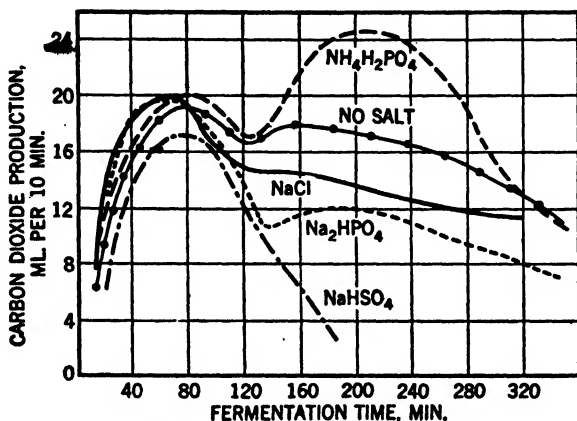


Fig. 10.—Effect of various salts on the rate of gas production in fermenting doughs. The salts were employed at a concentration of 0.02 equivalent (based on the anion) per 100 grams of flour (140).

more extensive treatment of yeast fermentation in breadmaking the review by Atkin, Schultz, and Frey (9a) may be consulted.

#### 4. *Amylase Action during Fermentation and Oven Baking*

As already noted, flours milled from sound wheat contain appreciable quantities of free and bound  $\beta$ -amylase. The normal amylase activity of the flour is frequently supplemented by the addition of malted wheat flour during the milling process or by the inclusion of diastatic agents, such as diastatic malt, in the baking formula. In addition, various commercial preparations of partially degraded starches or dextrans are often added to increase the quantity of readily available substrate.

It has been previously pointed out that the amylase activity in flour-water mixtures is limited by the small quantity of susceptible starch which is normally present in wheat flours. In unsupplemented doughs made from

sound flours, the saccharification is limited to that produced by  $\beta$ -amylase. Alpha-amylase, however, attacks damaged starch much more vigorously and produces new points of attack for  $\beta$ -amylase, so that under the combined influence of the two enzymes, rapid saccharification occurs. Also,  $\alpha$ -amylase, or an enzyme associated with it, is capable of attacking starch which is not sufficiently damaged to be susceptible to  $\beta$ -amylase; in fact, there is evidence that undamaged granules are slowly attacked, although the rate of sugar production from this source would appear to be too slow significantly to augment the sugar content of the dough.

In view of the foregoing, it is not surprising that  $\alpha$ -amylase is the component which is responsible for the increase in flour maltose value, and the

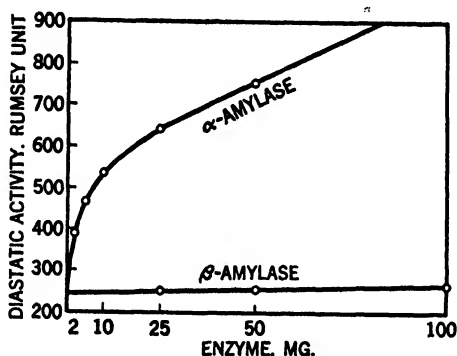


Fig. 11.—Effect of additions of  $\alpha$ - and  $\beta$ -amylase preparations on maltose value (diastatic activity) of flour (220). The quantities of enzyme are in terms of milligrams of the dry preparation added per five grams of flour.

increases in gas production in doughs, in dough mobility, and in loaf volume which are brought about by malt supplements. This has been demonstrated by several workers (72, 132, 172, 200, 220).

That normal flours contain sufficient quantities of  $\beta$ -amylase and that an increase in this component does not have any material influence on flour maltose value is shown in Figure 11 from Stamberg and Bailey (220); on the other hand, only small quantities of  $\alpha$ -amylase are necessary to produce a considerable increase. Similar results were obtained in a study by Kneen and Sandstedt (132) of the effect of additions of  $\alpha$ - and  $\beta$ -amylase on the gas production in fermenting doughs. It seems quite probable that the latent  $\beta$ -amylase of the wheat flour and malt supplements would be liberated by the proteolytic enzymes of the flour and malt during dough fermentation.



The decrease in hydrogen-ion activity which occurs as fermentation proceeds, doubtless increases amylase action in doughs, and it is fortuitous that the production of maltose is accelerated during the later stages of fermentation, since a high rate of gas production is essential during the proof period and the first few minutes in the oven in order to obtain an optimum loaf. The relative importance of  $\alpha$ - and  $\beta$ -amylase supplementation in this connection is shown in Figure 12 from Stamberg and Bailey (220). The doughs were baked without sugar in the formula in order to eliminate any

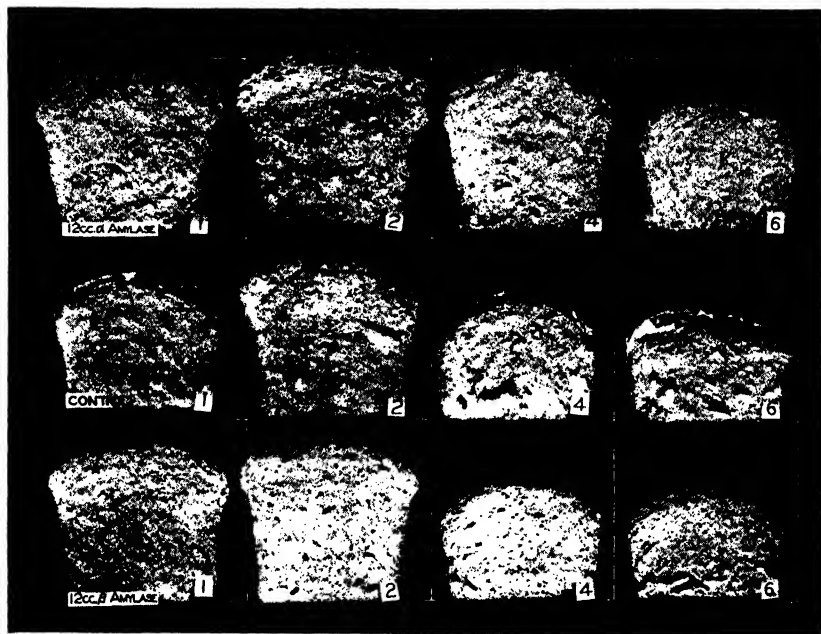


Fig. 12.—Relative effect of  $\alpha$ - and  $\beta$ -amylases in maintaining loaf volume after 1, 2, 4, and 6 hours (220). Doughs were baked without added sugar.

possibility that the effect of the amylases might be obscured. The addition of  $\alpha$ -amylase was more effective than  $\beta$ -amylase in maintaining the loaf volume upon prolonged fermentation.

The mechanically injured wheat starch is highly hydrated and contributes materially to the flour absorption (2, 200). Degradation of this fraction by  $\alpha$ -amylase produces dextrins of lower water-retaining capacity than the original material. As a result of this liquefying action, followed by the saccharification of the dextrins by the  $\beta$ -amylase which is present in

abundance, doughs which contain excessive quantities of  $\alpha$ -amylase tend to slacken and become sticky. To compensate for this, such doughs have to be made with a lower absorption. In doughs deficient in  $\alpha$ -amylase, less starch is converted and a limit-dextrin representing approximately 40% of the starch which is degraded remains, so that there is relatively little change in dough mobility under these conditions.

After doughs have been molded and panned, they are "proofed" by allowing the fermentation to continue in the pans until the dough attains a selected volume, usually that corresponding to the top of the baking pan. It is then placed in the oven. During this period, the rate of gas production must be at a high level in order to obtain bread of satisfactory volume, crust, and crumb characteristics. As the temperature of the dough rises in the oven, the activity of the various enzymes is at first greatly accelerated. Heat inactivation subsequently occurs.

The extent of starch degradation which occurs in oven baking will depend upon the rate at which the temperature of the dough rises, the hydrogen-ion activity of the dough, the concentration of calcium ions, and the  $\alpha$ - and  $\beta$ -amylase concentration. Bailey and Munz (12) have shown that the temperature of the interior of 500-g. loaves baked at 200° C. rises to approximately 60° in the first ten minutes of baking, and reaches a temperature of 70° in an additional two to three minutes. The hydrogen-ion activity of bread doughs at the end of the proof period is normally between pH 5.0 and 5.5, so that the acidity is favorable for amylase action. Bread doughs are made with tap water, and flour improvers containing calcium salts are extensively used so that calcium ions, which stabilize  $\alpha$ -amylase but have an inactivating effect on  $\beta$ -amylase (133), are normally present. In view of the presence of calcium ions and the relatively high thermostability of  $\alpha$ -amylase as compared with  $\beta$ -amylase, it is not surprising that  $\alpha$ -amylase activity is the more pronounced during baking. Wheat starch begins to gelatinize at a temperature of 65° to 68° C. (3), and this is accompanied by a marked increase in its amylolytic susceptibility. The marked liquefying effect of an  $\alpha$ -amylase (taka-diastase) on wheat starch suspensions as they are raised in temperature at a rate corresponding to that which takes place in oven baking is shown by the amylograph curves in Figure 13, from Anker and Geddes (8). When malt is added to flour suspensions, similar reductions in the peak viscosities of amylograph curves have been noted (72). Kosmin (124) has shown that bread contains more water-soluble intermediate products of starch hydrolysis than the dough made from flour milled from sprouted wheat. Freeman and Ford (72) found that bread baked from dough containing malt had a much higher dextrin

content than the bread from control doughs. While maltose markedly increased during baking, the quantities produced were essentially the same whether or not malt was present. These researches establish that the

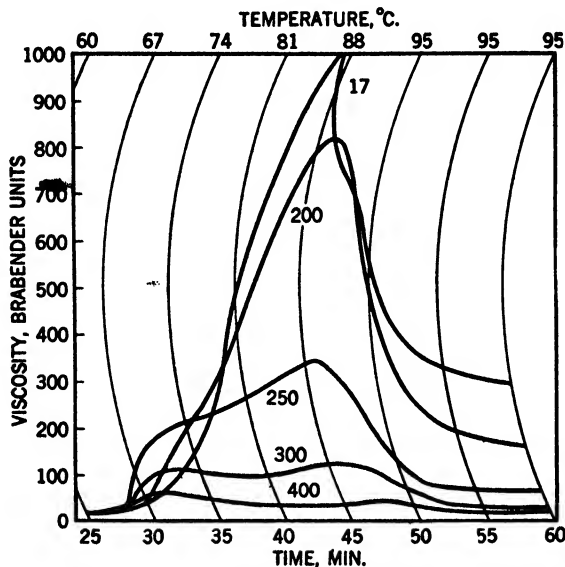


Fig. 13.—Amylograph curves for commercial wheat starch brought to maltose values of 17, 200, 250, 300, and 400 units by addition of a taka-diastase preparation (8). The starch suspensions contained 11.8% starch and the pH was 4.3.

activity of  $\alpha$ -amylase is of greater significance than that of  $\beta$ -amylase during oven baking.

## VIII. Significance and Control of Amylase Activity in Breadmaking

### 1. Significance

From the foregoing discussion it is clear that the  $\alpha$ -amylase activity must be kept within certain limits if optimum bread is to be obtained. An adequate level of  $\alpha$ -amylase activity is essential in order that gas production may be maintained at a sufficiently high rate to leaven the dough, and to provide a residue of sugar to give appetizing bread with a desirable crust color. Kneen and Sandstedt (132) have pointed out that even though the sugar content of doughs be adjusted so that the rate of gas production during the proof period is maintained at an adequate and constant level, the

addition of increasing increments of  $\alpha$ -amylase to a dough deficient in this component results in an increase in loaf volume. This is indicative of the fact that the dextrins produced during baking modify the gas-retaining properties of the dough.

Reference has already been made to the fact that doughs containing excessive amounts of  $\alpha$ -amylase (due to oversupplementation with malt, or to the use of flours milled from sprout-damaged wheat) become soft and sticky as fermentation proceeds. This was attributed to protein hydrolysis by the earlier workers (211) but in 1933, Kosmin (124) advanced the theory that the production of sticky doughs, as well as a moist crumb in the baked bread, was due to liquefaction and dextrinization of starch. This view is supported by the work of many subsequent investigators. Thus, Read and Haas (189) found that extracts of malt preparations, which had been treated with safranine to remove proteases, were still able to produce stickiness in doughs. Employing purified  $\alpha$ - and  $\beta$ -amylase preparations in which the protease content was reduced by safranine, Munz and Bailey (172) found that changes in dough mobility were obtained only with extracts containing  $\alpha$ -amylase, and that when this enzyme was inactivated by acid treatment, the extracts had no significant effect on dough properties. Geddes, Hildebrand, and Anderson (77) showed that when a series of malted wheat flours of widely varying proteolytic activity were added to a common untreated base flour in amounts sufficient to produce flours of equal gassing power; the resulting flours produced doughs of similar handling properties and the breads did not differ significantly in loaf volume or other characteristics. In no case did the malted wheat flour account for as much as 5% of the proteinase activity of the flour blends. These observations were confirmed by Hildebrand and Burkert (97). They also showed that the addition to fermenting doughs of increasing increments of extracts of malted wheat flour, from which the proteases had been partly removed by precipitation with safranine, caused progressive increases in dough mobility and stickiness. On the other hand, the addition of safranine precipitate to doughs was without any material influence on these dough properties. These researches support the view that the effect of excessive dosages of malted wheat flours on dough mobility and stickiness is to be ascribed to their  $\alpha$ -amylase activity rather than to proteinase activity.

During oven baking, excessive liquefaction and dextrinization of starch are responsible for the production of bread with a moist doughlike crumb of poor eating quality because of its decreased ability to bind water set free by coagulation of the gluten proteins (72, 124, 169, 170). The correct level of  $\alpha$ -amylase activity appears to be of particular importance in the manu-

facture of breads made largely from rye flours, as is common in Scandinavia and Northern Continental Europe. According to Molin (169, 170) and Brabender, Mueller, and Köster (31), variations in the amylase activity of rye flours exist which result in wide differences in bread quality. Insufficient  $\alpha$ -amylase is characterized by a dry, brittle crumb and the crust becomes cracked and torn on cooling, whereas an excess results in bread with a soggy, doughlike crumb which frequently pulls away from the crust leaving large hollow spaces. Kent-Jones and Amos (118) have pointed out that excess  $\alpha$ -amylase activity is of particular significance in bread doughs that are baked slowly.

Sprout damage of wheat and rye, and consequently excess  $\alpha$ -amylase activity, is much more common in the insular climates of the British Isles, Scandinavia, and Northern Continental Europe than in the more arid grain-producing areas of Argentina, Australia, and the Great Plains region of the American continent. In fact, high protein plump wheats are prone to yield flours of low gassing power, particularly in years when the rainfall is comparatively light during the latter part of the growing period. It is therefore not surprising that American cereal technologists have been concerned primarily with overcoming deficiencies in  $\alpha$ -amylase activity, while the European workers have devoted considerable attention to a study of the undesirable effects of excess  $\alpha$ -amylase activity and the development of convenient methods for its detection.

## 2. *Methods of Increasing $\alpha$ -Amylase Activity*

The production of bread of uniform quality from day to day requires that the flour, yeast, and other baking ingredients be of standard and uniform characteristics so that the various steps in the baking process can be carried out in a regular manner. American bakery practice and the quality demands of the consumer in the United States dictate the use of strong, relatively high protein flours produced from hard wheats for breadmaking purposes. Warm, dry weather during the filling and maturation of the wheat kernel favors the production of high-protein grain. As these conditions are also conducive to the production of wheats yielding flours of low gassing power, strong flours of high potential breadmaking value frequently fail to yield satisfactory bread unless precautions are taken to provide adequate gas production during baking. Although it is customary to include up to 6% of sugar in American bread formulas, it must be emphasized that a very large proportion of the bread is made by the sponge and dough process. As the sugar is not added until the dough stage, the sponge fermentation may be limited by the deficiency in fermentable sugars unless the gassing

power of the flour is sufficiently high. It is now the general practice of flour millers to maintain the maltose value or gassing power of the flours they produce at a level which is adequate to preclude yeast starvation under the usual systems of baking.

Inclusion of a small percentage of sprouted wheat in the mill mix was the first method used by the miller to increase flour-gassing power (181, 225), but the  $\alpha$ -amylase activity of such wheat varies widely depending upon the extent of the sprouting, as influenced by moisture, time, and temperature, so that very variable results are obtained with different lots. Moreover, sprouted wheat is not always available to the miller and its inclusion in the mill mix brings about a greater increase in the amylase activity of the low-grade flour streams, which are of relatively high gassing power, than in the highly purified streams which enter into the patent flours and where the supplementation is most needed. For these reasons, millers, when necessary, now usually feed small percentages of malted wheat flour or malted barley flour into the various grades of flour, thereby permitting the independent adjustment and control of the  $\alpha$ -amylase activity of each grade to the desired level. The definitions and standards of identity for flour, effective January 1, 1942, under the Federal Food, Drug, and Cosmetic Act of 1938, permit the use of malted wheat, malted wheat flour (not exceeding 0.5%), and malted barley flour (not exceeding 0.25%), singly or in combination, to compensate for any natural deficiency in amylase activity (65). In American bread flours, it is customary to bring the flour maltose value to 280-350 units, as determined by the Blish and Sandstedt procedure (20), the highest levels being employed with high protein flours used in hearth baking.

Although the malts of barley and wheat are the only supplements which are permitted in the current definitions and standards of identity for flour, Kneen (125) has shown that the germination of maize, oats, rye, and sorghum also leads to a pronounced increase in  $\alpha$ -amylase activity, the various germinated cereals differing principally in their  $\beta$ -amylase activity. Molds, such as certain strains of *Aspergillus oryzae*, usually grown on cooked wheat bran, produce an amylase of the  $\alpha$ -type. Bacterial amylases, produced by selected strains of *Bacillus subtilis* or *B. mesentericus*, are of the  $\alpha$ -type and are characterized by higher thermostability than malt  $\alpha$ -amylase (17); because of their high thermostability, such preparations would likely be unsuitable for use as a malt supplement since they would undoubtedly bring about excessive liquefaction of the starch during baking. The animal amylases also produce a type of starch degradation similar to that of  $\alpha$ -amylase from the cereals. Pancreatic amylase, which is of lower

thermostability than cereal  $\alpha$ -amylase and has an optimum hydrogen-ion activity in the region of pH 7.0, has been reported by Green (82) to give satisfactory results as an amylase supplement in breadmaking.

### 3. Evaluation of Malt Supplements

Since both the desirable and undesirable effects of amylase supplements in breadmaking depend primarily on their  $\alpha$ -amylase activity, the Lintner value of a malt, which is based on its ability to convert gelatinized starch

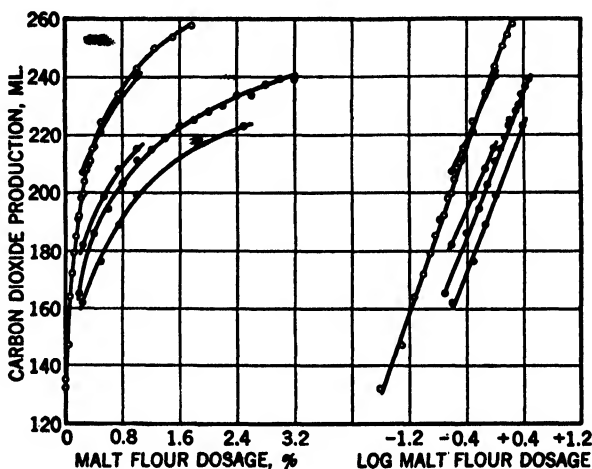


Fig. 14.—Relation between malt flour dosage and gas production of fermenting doughs (no added sugar) prepared from the flour blends (98). The different curves are for the addition of malted wheat flours of varying activity.

to maltose and is largely a measure of its  $\beta$ -amylase activity, is of no utility in evaluating malts for breadmaking. Suitable methods include: direct determination of the  $\alpha$ -amylase activity; measurement of the increase which malts bring about in flour maltose values, in dough gas production, or in starch liquefaction; and finally, determining their effects on the properties of bread baked under controlled conditions. Although the properties of the bread are the ultimate criteria, the baking test is unsuitable for routine work and it is used primarily to establish desirable numerical values for the other types of measurements.

The method of Sandstedt, Kneen, and Blish (201), in which the dextrinogenic activity of the malt is measured in the presence of an excess of  $\beta$ -amylase, is an appropriate procedure for evaluating malt supplements since

it is specific for  $\alpha$ -amylase and is capable of giving reproducible results in different laboratories. Leatherock, McGhee, and Giertz (143) suggested that the relative activities of malted wheat flours could be estimated "by adding various percentages of the different malt flours to an untreated flour, and determining the maltose before and after treatment." Davis and Tremain (56) elaborated on this technique by comparing malt supplements in terms of the quantities to give a definite level of response. They showed that such estimates of "malt value" could be made by adding various increments of the material to be tested to a suitable untreated base flour, de-

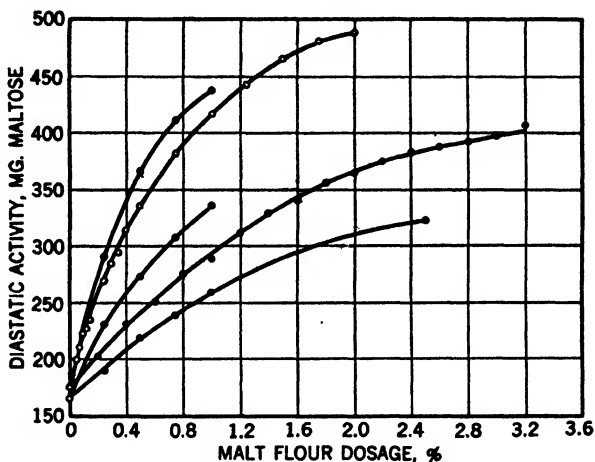


Fig. 15.—Relation between malt flour dosage and maltose value of the flour blends (98). The different curves are for malt flours of varying activity.

termining the maltose value or gas production, and estimating from graphs of the data thus secured the amount of malt required to give an arbitrarily selected level of either measure. This technique was found to be necessary since a curvilinear relation exists between malt dosage and flour maltose value or flour gassing power. Hildebrand and Geddes (98) showed that the gas production for any given malted flour blend varied directly as the logarithm of the dosage added to the base flour, whereas the relationship between dosage and flour maltose value took the form of a quadratic curve (Figs. 14 and 15). An estimation of the dosage of various malts required to produce a given level of gas production can therefore be more readily made from gas production data than from maltose values. Since flours vary in their response to malt supplementation, depending upon the susceptibility



of their starch to amylase attack, these autolytic methods provide relative, rather than absolute, results; it also follows that the quantity of a given malt required to raise the maltose value or the gassing power of a flour to a desired value can only be obtained by measurements with each flour under study. The same method may thus be employed for evaluating malts and for the adjustment of the amylase activity of a flour to the desired level. The utility of the "maltose increase" method of evaluating malts for bread-making has been confirmed by Freeman and Ford (72). Kneen and Sandstedt (132) obtained a correlation of 0.94 between the stimulation of gas production and the  $\alpha$ -dextrinogenic activity of malts, which indicates that increased gassing power is almost wholly dependent on  $\alpha$ -amylase activity. Meredith, Eva, and Anderson (166) obtained a correlation of 0.71 between these two properties in the instance of malts prepared from different varieties, and a correlation of 0.79 for malts representing wheats grown in different locations. It is therefore clear that a fundamental relation exists between  $\alpha$ -amylase activity and gas stimulation.

Another available technique for evaluating malts and controlling the required dosage in flour supplementation is based on the liquefying action of  $\alpha$ -amylase. Increments of the malt may be added to the flours under study and the effect on the peak viscosity of suspensions of the blends upon gelatinization may be determined by means of the Brabender amylograph. However, Anker and Geddes (8) have shown that such variables as hydrogen-ion activity, starch content, protein content, inherent differences in starch characteristics and in the extent of mechanical injury suffered by the starch during milling influence the maximum paste viscosity of suspensions of the unsupplemented flours, and thus would interfere with the interpretation of the relative height of the amylograph curve as a direct index of the  $\alpha$ -amylase activity of flours which differ widely in these characteristics. In mill control work, the interfering factors would not come into full play; the mill mix for the production of any particular type of flour represents a composite of certain restricted types of wheat and the protein content (and hence also, to a large extent, the starch content) is controlled within rather narrow limits. How closely maximum paste viscosity would be correlated with  $\alpha$ -amylase activity under such conditions remains to be definitely established, although the studies of Freeman and Ford (72) and of Brown and Harrel (38) indicate that this technique is useful for control purposes. Brabender, Mueller, and Heide (30) have reported that the amylograph gives a reliable index of the  $\alpha$ -amylase activity of flour used in making Iris soda bread where inferior crumb characteristics are frequently encountered.

Reference has previously been made to the studies of Kosmin (124) which showed that flours milled from sprouted wheats yield bread containing a much higher percentage of water-soluble nonnitrogenous substances than normal flours. Kent-Jones and Amos (118) have employed a method involving the determination of a "dextrin figure" on the water extract of a flour suspension autolyzed at 62° C. for 30 minutes. By classifying the results on a series of flours into groups according to the crumb characteristics of the bread baked from the same samples, it was possible to set up approximate limits within which the dextrin figures should be held to avoid inferior crumb characteristics under different conditions of baking. The significance of amylase activity in breadmaking and the evaluation of malt supplements are more exhaustively treated in a recent review by Kneen and Sandstedt (132a).

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# TOCOPHEROL INTERRELATIONSHIPS\*

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### I. Classification of Vitamin Activity

Unraveling the part that each vitamin plays in the animal economy has brought investigators to the possibility of cooperative action in pairs, in threes, and, ultimately, all together. Does one vitamin modify another's activity? The day of individual vitamin discoveries, though not over, is in deepening twilight. The emphasis now is in finding the places and quantitative significance of all the vitamins in body metabolism. What is the exact chemical process by which each vitamin is utilized? In the larger sense, what is the efficiency of transfer from field to kitchen, to ultimate body site in health and disease?

Vitamins are constituents or modifiers of enzyme systems (55, 161). For if, as a broad generalization, enzyme is the name given to organic molecules that catalyze metabolism, and if a vitamin is essential for individual acts of metabolism, then a vitamin *ipso facto* plays an enzymic role—primarily, as the enzyme itself or as part of a prosthetic group of an enzyme, or, secondarily, as an agent that modifies the efficiency or yield of an

\* Communication No. 80 from the Laboratories of Distillation Products, Inc.



enzymic process. We list the following activities in which a vitamin may engage:

1. Primary—the vitamin cooperates.
  - a. The vitamin is an enzyme.
  - b. The vitamin is part of an enzyme molecule.
  - c. The vitamin cooperates within an enzymic reaction.
2. Secondary—the vitamin\* modifies or is modified.
  - a. The vitamin preserves another vitamin or enzymic agent from destruction — sparing action or agent.
  - b. The vitamin accelerates destruction — spending action or agent.
  - c. The vitamin replaces or is replaced by another vitamin or anti-vitamin — antivitamin or replacing agent.
  - d. The requirements for the vitamin are altered by nutritional factors, not themselves vitamins.

The most fundamental information needed about any vitamin is the nature of the complete enzymes in which it participates; but, practically, that may not be as useful as a knowledge of sparing agents with which it is involved. For thiamin, the ultimate function is exerted in class 1*b* as a constituent of carboxylase which, in turn, is concerned with the metabolism of carbohydrates through pyruvic acid (100). This is the central scientific fact but it is not one which, in the present state of knowledge, can be manipulated. On the other hand, since thiamin is concerned chiefly with carbohydrate metabolism, replacing carbohydrate with fat in the diet lessens the minimum requirement of thiamin (5), and we say that "fat spares thiamin" (an interaction listed as 2*d* above). This fact, though less basic, evidently can be manipulated, for quantitative knowledge would enable the authorities to place the minimum adequate allowance of thiamin in flour for a ravished nation.

Again, consider the case of niacin, which, with adenine and phosphoric acid, is known to participate in the important enzymes, coenzyme I and II (161), another cooperation in class 1*b*. But, unit quantity of niacin does not always produce unit protection from pathological deficiency. A minimum quantity fed, as contained in skim milk, to dogs prevents black tongue, but if fed as yellow corn the dogs become sick (95). Another minimum quantity supplied in the usual poultry feeds gives good growth for chicks. Fed as yellow corn it fails to give growth, but growth is restored if a minute quantity of tryptophan is supplied (96). The fundamental ques-

\* Other substances such as unsaturated fatty acids, peroxides, essential amino acids, proteins, and, indeed, the whole balance of the diet must be included in this role.

tion of whether tryptophan and niacin cooperate according to class 1c, or whether secondary effects under class 2a-d are involved, remains unanswered, but the important practical point emerges that there are gross diets and trace dietary factors which are synergistic, favorably or otherwise, with niacin.

If we admit the importance of synergism, where, legitimately, can the synergy be considered to start? In tissue, in the circulation, in the gut wall, or stomach; and, if the latter, in food, in cooking, in factory processing? The problem is simplified because many of the factors which exert the most powerful secondary synergies within the animal are responsible for the protection or destruction of labile nutrients before the food reaches the animal. As a *practical* measure, therefore, we are concerned with the mutual interaction of the vitamins from their very genesis in the plant to the ultimate molecular stage of utilization by the animal. The more steps or classifications into which the journey can be divided, the more chances there are of improving the "total-journey yield" of each vitamin. This must be our excuse for extending the classification from the vital to the economic. Many persons are busy devising systems of nomenclature for the synergies; and it was little surprise to find Dr. H. J. Almquist speaking in this vein in discussing the interrelationships among choline and other compounds having the functions of choline in part (3).

The word "synergy," from Greek roots, implies *combined* action and not necessarily *increased* action. To the vitamin assayist, a synergism is noted as an *increase* in the activity of a dietary element. Feeding vitamin E with carotene, fat with thiamin, or tryptophan with niacin provides an increased response to the second members. To accept this statement at its face value would lead us to an entirely wrong point of view. To the best of our knowledge, if we exclude synthesis by intestinal microorganisms there are no cases of real enhancement of potency, only *repression of antagonism*. If a quantity, *q*, exactly, of a vitamin is required to participate in a metabolic change at a local body site, and if each molecule were to be delivered into combination without fail by a Maxwell Demon, the potency would be unity, and the vitamin 100% efficient. But if some molecules decay in the food, others during digestion, if some fail to penetrate the gut wall or find transportation to the body site, and if some of the survivors fail to link up with their alter egos to complete the enzyme, the efficiency will be far, far below the 100% mark. Any agent which facilitates passage or guards the vitamin during this total journey will appear to enhance the potency and is likely to be mistaken for a positive synergist.

At the risk of redundancy, we present the classification of synergies in



pherols, ascorbic acid, etc.), and accelerated by bacterial change, rancidifying agents, and so forth.

*Step 2. Transport and storage.* The losses from this step are often extremely serious, but since their incidence and prevention have been much studied, they are referred to only in Figure 1.

*Step 3. Liberation.* Breaking open the living cell, hydrolysis of glucoside or ester, conversion of provitamin to active form.

*Note:* Some of these steps may take place later during digestion, step 6, or body distribution, steps 7-8.

*Step 4. Commercial handling and cooking.* This includes refining, cooking, compounding in food and medicine, exposure to shelf life by vendor and purchaser. Examples readily come to mind: the deterioration of A vitamins during passage from fresh whole milk through sour cream and the butter churn to the condition, sometimes, of a rancid pat on the plate of the consumer; or the loss to various extents of all vitamins in alfalfa and corn silage in the steps between reaping and ultimate ingestion by the cow.

*Step 5. Ingestion.* Includes simple mastication and chewing the cud.

*Step 6. Digestion.* Exposing to acid and alkaline digestive juices, aeration (massive aeration with ruminants), enzymic, and other chemical changes.

*Step 6a. Rejection.* Some of the digested nutrients are rejected in the feces. This can be considered as a branch of either step 6 or 7 and is marked 6a. Carotene and tocopherols are regularly found in the feces in quantities roughly proportional to intake, so that correction must be made for loss (57, 74, 131).

*Step 7. Diffusion through the gut wall.* This process is, in part, the positive restatement of step 6a. There may be failures of the nutrient to diffuse as, for instance, vitamin A, carotene, or tocopherol in steatorrhea, leukemia, celiac disease, disturbances of the biliary function, or in acute colitis. Simultaneous ingestion of phospholipides (2) or bile salts (54) will increase absorption through the gut wall and *these substances will play the part of vitamin synergists*. Further modifying the efficiency of transfer will be chemical losses, for instance, during the hydrolysis and recombination of esters (53).

*Step 8. Conveyance.* The vitamin is conveyed from the gut wall by blood and lymph to redistribution centers and storage depots and thence to the functional body sites. This journey requires a *carrier*. For the water-soluble vitamins, this is water and there is unlikely to be any shortage of carrier. But for the oil-solubles, the carrier must be a specialized adjunct of the vitamin and necessary for its proper distribution—for instance,

a suspended lipide or a phospholipide or protein complex. *In the absence of sufficient lipide carrier, the efficiency of conveyance will be depressed.* Suggestive evidence concerning this point will be presented later.

*Steps 7a and 8a. Excretion and destruction.* Vitamins C, B<sub>1</sub>, and B<sub>2</sub> are continuously excreted in the urine, while vitamin A is destroyed by the Kupffer cells in the liver (126). Vitamins B<sub>2</sub>, K, etc., may be synthesized by intestinal flora. Evidently, these quantities modify the net amounts reaching the ultimate body sites. Any substances or habits of life which modify excretion or destruction have the quality of vitamin synergists.

*Step 9. Acceptance.* A potentially active chemical that is cruising within the body channels without being chemically linked with functional processes can hardly influence or control such processes, any more than a substance that is transparent to radiation can be affected by that radiation. A vitamin, therefore, must be *accepted* by tissue or other functional entity as an adsorbate or by direct chemical interaction. This step may be merely one of anchorage preparatory to chemical conversion to the prosthetic group or enzyme (step 10), or it may be identical with step 10. That such acceptance or anchorage is a reality may be inferred from the analysis of various organs. Vitamin C is concentrated in the adrenals (168), vitamin A in the liver and eye (157), vitamin E in heart muscle and mammary gland (107), riboflavin in liver and kidney (156), and so on. *If the mechanism of acceptance is faulty, the efficiency of vitamin utilization will be impaired.* Symptoms typical of the vitamin deficiency will ensue but feeding the vitamin may not relieve the deficiency. Indeed, an excess of the vitamin administered today may use up tomorrow's quota of fixative and precipitate an enhanced deficiency. Under such circumstances, giving too much of a vitamin for too long would induce the deficiency syndrome of that vitamin. The clinician is familiar with this general picture: a patient who exhibits all the symptoms associated with lack of a particular vitamin and responds favorably at the start of vitamin medication but soon relapses into a condition more acute than before. In such cases, the possibility that there is a shortage of carrier or cooperating agent should be considered.

*Step 10. Enzyme formation.* Finally, the vitamin must enter into combination with the operative enzyme. The simpler steps of such processes are well established for the water-soluble vitamins: thiamin passing to the pyrophosphate in cocarboxylase, riboflavin to the phosphoadenine dinucleotide, and niacin to the diphosphopyridine nucleotide of coenzyme I. The elaborations of biotin, pyridoxine, and pantothenic acid are being widely explored at the present time. For all these interactions, there are

two general requirements: a supply of component parts—phosphoric acid and suitable protein in carboxylase, for instance—and a catalyst to promote their interaction. In most cases the enzyme itself is the catalyst or rather, the total enzymic cycle is self-perpetuating as, for instance, when phosphopyruvic acid rephosphorylates thiamin (6). In other cases the components are numerous. Thus, Lipmann (100) finds that five entities—(1) cocarboxylase, (2) flavin-adenine-dinucleotide, (3)  $Mg^{++}$ ,  $Mn^{++}$ , or  $Co^{++}$ , (4) protein, and (5) inorganic phosphate—are needed for the oxidative decarboxylation of pyruvic acid by *Bacillus delbrueckii*. Evidently, shortage of the metal or phosphate ions could limit this reaction as thoroughly as shortage of the vitamin components themselves. Both in the initial formation of an enzyme and in its operative re-use, there is the chance of failure, and an efficiency factor must be attributed to this step 10 which is less than unity, and in faulty nutrition of bacterium, animal or man may be very low indeed. Those factors, other than the vitamin itself, which modify step 10 must be considered as synergists. In the classification proposed by Almquist (3) this type of synergist would take first place, the preservative sparing agents being classed as secondary synergists. We concur with his rating.

Little is known about the operative enzymes of the oil-soluble vitamins. Although more than a thousand enzyme systems are already postulated the identified vitamins number less than thirty. The better understood water-soluble vitamins have each been found to participate in many different systems; and the fewer oil-soluble vitamins are likely to be discovered participating in many times their own number of enzyme activities. This anticipated multiplicity of function only accentuates our lack of knowledge.

True, vitamin A has been found combined with protein in rhodopsin (158) of the visual cycle, but nothing is known of the A prosthetic group which promotes the differentiation of epithelial cells, the proper formations of the eye (162), etc. Some vitamin A is carried in the blood as fatty acid ester, and a smaller more variable quantity, as the free alcohol (25). What determines the ratio, what decides the balance between depot stores and the cruising fraction, and what portion of this fraction, free or combined, is used physiologically, is unknown. It is significant that carotene, which appears to have no vitamin activity except vicariously as a precursor of vitamin A, fluctuates in concentration in the blood plasma in almost direct reflection of oral intake, whereas the concentration of vitamin A in the plasma is only slightly affected by gross alterations of intake, *if these are maintained over long periods* (153). Single large doses of vitamin A elicit an immediate plasma response (24).

The final steps through which the D vitamins operate remain obscure in spite of the mass of data accumulated concerning the influence on bone formation and varying balance of calcium and phosphorus. These two inorganic ions, incidentally, must be recognized as powerful D synergists (139), classification undecided.

The cycle of vitamin K, prothrombin, thromboplastin, and calcium ions is well understood, but there is a gap in our knowledge concerning how the vitamin initiates the cycle in the liver—steps 9 to 12 for vitamin K are unknown (103). Evidently calcium and thromboplastin should be classed as cooperators or synergists. When vitamin K alone is absent, the simple hemorrhagic syndrome is produced, curable with vitamin K. When the rest of the cycle is deranged, the complicated syndrome of hemophilia results which does not respond to vitamin K. There is an almost exact parallel between these conditions and primary fibrositis, which responds to vitamin E, and progressive muscular atrophy, which does not.

The E vitamins,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols, occupy a unique position in the classification. They are perhaps the most versatile and active of all the vitamins in steps 1-8 and perhaps 9, but only  $\alpha$ -tocopherol, the fully methylated member, appears to participate in step 10, enzyme formation. The activity of  $\gamma$ -tocopherol is variable according to species and dietary conditions, and would seem to be quantitatively equal to the animal's ability to effect methylation (155) to the  $\alpha$ -variety. If this be so, the statement that  $\gamma$ -tocopherol has one-twelfth the activity of  $\alpha$ -tocopherol would mean that the E-depleted white rat can convert one molecule in twelve to  $\alpha$ -tocopherol (perhaps one in from sixteen to twenty, if correction is made for the sparing action of the large quantity of  $\gamma$ -tocopherol fed) (74). An alternative explanation of the poor efficacy of  $\gamma$ -tocopherol would be poor absorption through the gut wall. There is, however, no evidence for such an assumption.

As to the mechanism by which  $\alpha$ -tocopherol is carried (step 9), fixed (step 9), and utilized (steps 10-12), almost nothing is known, and the extent of our ignorance forms much of the text for later sections.

## 2. Summary of Classification

Each stage of utilization presents opportunity for loss of vitamin or failure to react, and to each stage can be attributed a fractional coefficient,  $f$ . Thus, the maximum generated quantity of vitamin,  $Q$ , is related to the smaller quantity,  $q$ , which is finally metabolized:

$$q = f_1, f_2, \dots f_{12}Q$$

The factors  $f_6 \dots f_{12}$  together make up the coefficient of physiological utilization,  $\psi$ , developed for vitamin A in a previous article (72). We may now group the preceding factors  $f_1 \dots f_5$  as the coefficient of economic utilization,  $\phi$ , whence:

$$q = \phi \cdot \psi \cdot Q$$

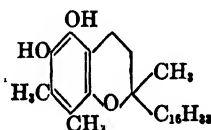
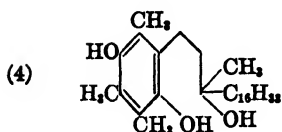
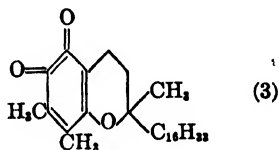
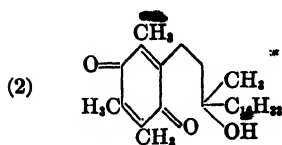
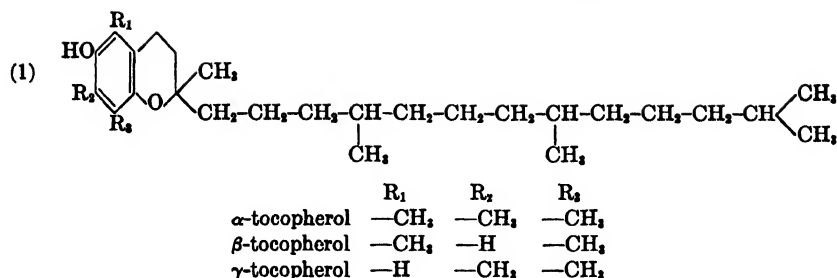
As far as we know at present,  $\phi$ , and perhaps  $\psi$ , are influenced more by physiological antioxidants than any other single factor. Physiological antioxidants all merit the rating of secondary vitamins, to which the terms covitamin, sparing agent, and synergist are alternatives, though not necessarily exact synonyms. Chief among the covitamins and physiological antioxidants are the tocopherols, themselves augmented by vitamin C, some B vitamins, and the natural phenols.

## II. Vitamin E and Covitamin E

The classification of the tocopherols with the fat-soluble vitamins is anomalous, for although they are soluble to the extent of complete miscibility with fat, they occur in the animal body associated chiefly with protein. In contrast to vitamin A, which accumulates in the liver, the highest *normal* concentrations of E appear in the spleen, lung, and muscle of the heart. In the vegetable world, where the tocopherols are created (the manufacture of vitamin E by animals or bacteria has not been reported), the association is again with the cytoplasm rather than the oil depots. Plants which are extremely low in oil still require a normal complement of vitamin E. Celery oil, for instance, contains 1.5% of vitamin E, which may be compared with 0.3% in wheat-germ oil. In its primary function as an essential component of tissue, vitamin E behaves as a water-soluble vitamin that requires a lipide carrier for transport. Only in its secondary function as an antioxidant does it generally behave as a truly fat-soluble vitamin.

The primary function of  $\alpha$ -tocopherol is unknown, but the secondary physiological actions of all three tocopherols appear to follow logically from their chemistry. All possess a chroman structure exhibiting the properties of pseudohydroquinones, in which one of the oxygen atoms is part of a bridge in the second ring (Formula 1).  $\alpha$ -Tocopherol oxidizes directly to the *p*-quinone (30) (Formula 2), with consequent fission of the second ring.  $\gamma$ -Tocopherol retains the second ring and oxygen bridge intact on mild oxidation and gives an *o*-quinone (148) (Formula 3). Both quinones are readily reducible to the corresponding true hydroquinones (30, 136) (Formulae 4) which, of course, are not identical with the E vitamins.





$\alpha$ -Tocopheryl hydroquinone is restored to  $\alpha$ -tocopherol in the presence of strong acids (HBr,  $\text{H}_2\text{SO}_4$ , etc.), but it is not known whether the conversion occurs *in vivo* to any extent (30, 150).

Natural vitamin E differs from all other vitamins in that its fragments and immediate conversion products are probably active secondary vitamins. In thinking of vitamin E, as it occurs in nature, we must consider a complex mixture with some of the components in natural equilibrium, but each with differing function, or site of operation, as suggested in Table I—which lists information of a very approximate nature, some of it perhaps faulty, but the service that a correct table could perform is of the first importance. It is failure to appreciate the functional multiplicity of the E vitamins that has led to so much misunderstanding in the use of the vitamin.

Thus, in the vegetable world, the E vitamins are preserved chiefly in reduced condition, protected by the cell wall and stabilized with accessory covitamins of a kind so well elucidated by Mattill (112) and his associates (18, 51). In this manner  $\alpha$ -tocopherol and traces of fat are preserved in the germ of the wheat or the seed of the fruit, through the heat and cold of the seasons, the dry and damp of the weather, until propitious circumstance allows the seed to sprout. Oil, pressed from the soybean a year or

TABLE I  
FUNCTIONS OF THE TOCOPHEROLS AND TOCOPHEROL DERIVATIVES

Name	Status	Function	
		Physiological	Economic
$\alpha$ -Tocopherol	Primary	Indispensable component of tissue Antisterility Antidystrophy	
	Secondary	Antioxidant for tissue, fat, intestinal contents	Antioxidant
$\beta$ -Tocopherol*	Secondary	Antioxidant for intestinal contents	Antioxidant
$\gamma$ -Tocopherol	Secondary	Antioxidant for intestinal contents	Antioxidant
$\alpha$ -Tocopheryl <i>p</i> -quinone	Secondary	Bactericide, prophylactic, oral hygiene?	Antioxidants,† also oxygen carriers and rancidifying agents under appropriate conditions
$\gamma$ -Tocopheryl <i>o</i> -quinone	Unknown	Oxidation of carcinogens in the intestinal tract?	
$\alpha$ -Tocopheryl <i>p</i> -hydroquinone	Secondary	Intestinal antioxidant	Antioxidant
$\gamma$ -Tocopheryl <i>o</i> -hydroquinone	Secondary	Intestinal anticixidant?	Poor antioxidant

\*  $\beta$ -Tocopherol is the least plentiful of the E vitamins and little is known of its biochemistry.

† Though the tocopherols and their quinones are, surprisingly enough, equally potent antioxidants, *e. g.*, for vitamin A in oil solution, *in vivo* the varying affinity of tissue for the active —OH or =O, respectively, should determine their site of action.

more after harvesting, contains the tocopherols ( $\gamma$ -, with 10–20%  $\alpha$ -) in almost completely reduced form, together with the oil-soluble, co-protection agents such as lecithin and complex phenols (112). Ascorbic acid and certain B vitamins, also instrumental in stabilization, stay with the meal. Nature has attended well to her packaging problems.

The state of the E vitamins in the animal differs according to species and habits of eating. Swallowed, as in fresh lettuce, by man, the vitamin is ingested in reduced form and passes to various body locations where it remains in equilibrium with its quinones and their hydroquinones, as determined by the reduction potential of the media. Scudi and Buhs find 75% of the tocopherol in the blood plasma of the dog and approximately 30% in human blood in the quinone form (136). Vitamin E, ingested from alfalfa by the cow, will suffer some destruction during rumination and passage through the large sectional stomach. The physiology of the cow must evidently accommodate the tocopherol, in quinone form, more satisfactorily than does the human. The saving factor for the cow is the large quantity of tocopherols available from fresh pasturage. As a corollary,

the barn-fed cow, bred to give much milk, becomes acutely short of E vitamins. Even so, the damage to the tocopherols can be considered to have occurred chiefly before passage into the cow's system. The seed, the lettuce, the man, or cow can all be considered as protective media—mechanical and chemical—for the *in vivo* cycle of the tocopherols.

Contrast this with the fate of tocopherols in the *applied* economic cycle. Manufacturers of animal feeds, of milk and egg powders, and of reinforced vitamin products attempt to apply the tocopherols by spraying them in dilute solution onto the *outside surfaces* of the particles where, exposed to air, subject to surface catalysis, and unprotected by their natural congeners, they pass rapidly to quinones and worthless or harmful oxidation products. Added for the purpose of protective synergy, they reverse their role and become potent agents for destruction. That such crude measures are occasionally effective is due to luck, as when wheat-germ oil, added to milk before dehydration, passes into intimate combination with the fat-protein complex of the resulting powder, the stability of which is extended (101). A vitamin E concentrate, mixed with the diet in the assay of vitamin A by the U.S.P. method, is always ineffective (73); administered with the diet in standard vitamin E bioassays, it is generally less effective than when given in oil by dropper. With rats, cows, and humans, extra quantities of the vitamin—according to most recent developments—are best administered as a rich natural food or medicinally as a concentrate, a capsule, or by injection.

Another fact that might be deduced from Table I is the possible reversal of function by overdoses of vitamin E. If it be accepted from *in vitro* experiments that oxidized tocopherols can promote oxidations (120), then increasing the intake of vitamin E may cause secondary oxidative reactions which in turn will produce clinical symptoms. Suggestive also are Woolley's experiments with  $\alpha$ -tocopheryl quinone in displacing vitamin K (167a). Thus, covitamin E is a balanced oxidation-reduction vitamin and, like all balanced systems, it requires accurate quantitative treatment. What the correct quantities are in health and disease for each species and individual must be the subject of much future inquiry, but we shall refer to this aspect of the problem continually.

### 1. Primary Vitamin E Functions

The essential vitamin E function appears to reside in tissue and to be exerted by  $\alpha$ -tocopherol alone. Some experimental syndromes associated with impaired function are sterility in the rat, muscle dystrophy in the

rabbit and dog, and vascular system abnormalities in the chicken. Whereas the relative antisterility potencies of the three tocopherols have been measured with great care, study of dystrophy appears to be limited to a recent comparison of  $\gamma$ -tocopherol with  $\alpha$ -tocopherol for the rabbit (83) and Dam's report (27) that *dl*- $\beta$ - is one-sixteenth as potent as *dl*- $\alpha$ -tocopherol in preventing exudative diathesis in the chick.

**Tissue Slice Experiments and Basal Metabolism.**—The exact function of vitamin E as a component of tissue is still unexplained. Of the six known molecular species, only the dextrorotatory  $\alpha$ -tocopherol molecule appears to be indispensable. This specificity suggests involvement with an optically active endogenous reagent which could be an amino acid, then a protein, and finally tissue itself. Phosphoric acid has been postulated as an intermediate link (165). A critical analysis of normal and dystrophic muscle should throw light on the question. In experiments where vitamin E was fed for many months to dairy cows, the curd of the (acid) souring milk differed from usual milk curd, suggesting that  $\alpha$ -tocopherol had modified the nature of the casein.

A more direct approach to the problem than milk protein is the study of live muscle, which Houchin and Mattill (78, 79) and others (47, 123) have made with tissue slices in the Warburg apparatus. They have shown that the normal oxygen quotient of muscle is raised to 125–250% in vitamin E-deficient muscle and rapidly lowered to normal by perfusion *in vitro* with vitamin E phosphate. Muscle from the dystrophic animal fed  $\alpha$ -tocopherol a few hours before sacrifice has a nearly normal  $Q_{O_2}$  in the Warburg apparatus.

These variations of this tissue slice experiment are important, but those that still remain to be done leave the story pathetically incomplete. The relative activity of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols is virtually unexplored. So, also, is the expected depression of  $Q_{O_2}$  below normal by overdoses of diffusible tocopherols.

While the active and *basal metabolism* of the live animal are both conditioned by a multiplicity of factors, including, for instance, pituitary and thyroid activity and gross diet, it is evident that a factor such as  $\alpha$ -tocopherol which influences tissue respiration must affect at least basal metabolism. Reports on the rat indicate a depression of metabolism by  $\alpha$ -tocopherol (123), and a few unconfirmed experiments of ours on humans after prolonged high dosage with mixed tocopherols have shown severe curtailment of activity. If we take a teleological liberty, we may say that nature was faced with a problem. She required plenty of oxidation inhibitors to keep her inflammable empire safe from unauthorized com-

bustion, but she required a smaller, carefully metered amount of one of them for the activation and control of tissue;  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols, with their graded functions, were made to meet the demands.

**The Sterility Syndrome.**—The development of the condition (39) and its pathology and histology (105) have been described in great detail during the past seventeen years and will be mentioned here by inference only. The vitamin E bioassay test, based on prevention of resorption gestations, informs us of the median fertility dose (M.F.D.) of  $\alpha$ -tocopherol required by the suitably selected and depleted white rat. Quantities as high as 3 mg. were often mentioned (40) in early researches, but recently 1 mg. of *dl*-tocopheryl acetate has been accepted as the International Unit corresponding to the M.F.D. (84, 85). The current M.F.D. in the writers' laboratory is 0.6 mg. natural *d*- $\alpha$ -tocopherol, 0.9 mg. synthetic *dl*- $\alpha$ -tocopherol. Only the dextrorotatory portion of the synthetic mixture appears to have activity. The failure of the *levo* compound to participate is presumptive evidence that the *dextro* compound participates in a cellular enzyme reaction. The variation of M.F.D. from 3 mg. to 0.9 mg. shows that the utilization of the vitamin is incomplete and variable. Simultaneous administration of antioxidants, such as  $\gamma$ -tocopherol, *l*- $\alpha$ -tocopherol or diamylhydroquinone decreases the M.F.D. of *d*- $\alpha$ -tocopherol.

Another important attribute of the deficiency syndrome is that it is relieved by chemically equivalent quantities of the free  $\alpha$ -tocopherol and the acetate, phosphate, and palmitate. Since the esters are not intestinal antioxidants, the action must occur after absorption.

The antisterility equivalence of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols has been measured by many workers (89, 90, 108). The  $\gamma$ -compound has been found less and less active by succeeding workers, and we attribute this to the continued critical purification from traces of the  $\alpha$ -variety (164). The latest assay for  $\beta$ -tocopherol stands at four-tenths the activity of  $\alpha$ -tocopherol (65). Whether the relatively high activity is intrinsic or whether it represents failure to purify a chemical which so far has been available only in minute quantities has not yet been decided. We favor the idea that the activities of  $\beta$ - and  $\gamma$ -, which now stand at 33–40% and 1–10% of  $\alpha$ -tocopherol, represent the ability of the depleted rat to methylate the covitamins. If this is the explanation, rats fed diets high in methylating agents like choline and methionine should show a lower M.F.D. Chickens fed large quantities of  $\gamma$ -tocopherol should lay eggs containing enhanced amounts of  $\alpha$ -tocopherol. If, however, the lower potency is caused by faulty absorption or transport (steps 7 and 8), addition of bile salts, phospholipide, etc., should lower the M.F.D. Experiments to test these

hypotheses are under way. It is clearly important to determine whether  $\beta$ - and  $\gamma$ -tocopherols can participate, *per se*, in the primary enzyme reaction. The weight of the incomplete evidence from fertility experiments is that they cannot.

**The Dystrophy Syndrome.**—The loss of tonus and finally of gross elements of structure of the skeletal muscle of vitamin E-depleted rabbits is well known (123). There is an associated creatinuria which is often taken to mark the onslaught of the condition. Return of creatine excretion to normal is evidence of cure; at least it is a convenient indication when searching for curative compounds.

In spite of the care with which the syndrome has been studied, little interest seems to have been shown in the choice of pure tocopherol compounds, nor has there been conspicuous curiosity concerning the relative activities of the three tocopherols, perhaps because of the unavailability of pure specimens. Recently, Hove *et al.*, in this laboratory, have examined the effect of  $\gamma$ -tocopherol on the depleted rabbit (83). Creatinuria is relieved as rapidly as by  $\alpha$ -tocopherol and the cure of the dystrophy is eventually complete, but doses must be 5–10 times larger. A number of explanations can be devised for these facts and they merit consideration only by prompting experiments to settle the matter. The easiest explanation is that ten parts of  $\gamma$ -tocopherol will replace one part of  $\alpha$ -tocopherol in the constitution of muscle. The next is that the rabbit can methylate 8–10% of its intake of  $\gamma$ -tocopherol. The third is that the  $\gamma$ -tocopherol preserves from destruction that fraction of  $\alpha$ -tocopherol that still remains in the tissues of the animal, which is then redistributed under carefully “spared” circumstances. We favor a combination of the second and third views. These would account for the immediate cessation of creatinuria and would leave the central role of *d*- $\alpha$ -tocopherol unchallenged. The effectiveness of  $\beta$ -tocopherol in curing rabbit muscle dystrophy has not yet been examined.

**The Dystrophy Syndrome in Humans.**—Much of the failure (62) and frustration (31) that has arisen in medication with vitamin E may be traced to faulty clinical diagnosis. There are evidently both *simple* and *complicated* muscular deficiencies and there are also many symptomatically similar conditions that have nothing to do with vitamin E at all (117). Pains, aches, and stiffness due to neural derangement or to faulty uric acid or calcium metabolism will not yield to a vitamin that is concerned principally with tissue integrity. Steinberg distinguishes this clearly (142). The mild and therefore reversible form of human muscular dystrophy has been identified by Steinberg with “fibrositis.” The condition varies from



Fig. 2.—Photographs showing the effect of vitamin E on Dupuytren's contracture. The hands at the left display severe symptoms of the disease. On the right the same hands show improvement after three months' treatment with 250 mg. of natural tocopherols daily. (Courtesy of Dr. C. L. Steinberg and the Medical Clinics of North America, W. B. Saunders Co.)

simple pain in the arm, shoulder, or hip, with limitation of action but no discernible malformation, all the way to muscular pains accompanied by fibrous nodules, lipid sacs, or generalized swelling.

Recently, Steinberg treated successfully a number of cases of Dupuytren's contracture, the cause of which has hitherto remained unsuspected (142, 143). The symptoms comprise retraction of the fingers of the hand by acute permanent muscle spasm. The previous mode of treatment has been surgical, severing the affected tendons, but the condition was rapidly re-established with the contraction of the remaining tendons. Steinberg finds that 250 mg. tocopherols daily cause complete relaxation without resort to operation. Photographs taken before and after weeks of vitamin E supplementation are shown in Figure 2. Note that the patients have previously subsisted on normal diets from which they have been unable to derive sufficient tocopherols. In this syndrome, as in all others, the criterion of vitamin E deficiency is that the conditions are promptly relieved by *dl*- $\alpha$ -tocopherol acetate, wheat-germ oil, or mixed natural tocopherols. This is a *simple* human vitamin E deficiency.

There are two much more serious diseases, one incurable and the other fatal, which are believed to be due to lack of vitamin E or faulty vitamin E metabolism. *Amyotrophic lateral sclerosis* is caused by degeneration of the anterior horn cells in the lateral column of the spinal cord. The normal nerve tissue becomes replaced with scar tissue. *Progressive muscular atrophy*, on the other hand, is analogous to the experimentally produced dystrophies in the rat and rabbit. It involves the wastage of skeletal muscle but there is no sclerosis of the spinal cord. Partial cures and temporary remissions have been reported (163) for both diseases when vitamin E is given with thiamin and riboflavin, but the majority of evidence is that the diseases know no remedy (44). This has been attributed to the irreversible nature of the damage to the cell structure, confirming the observations of the histopathologists that tissue, acutely damaged by lack of vitamin E, is not amenable to repair by the vitamin. Recently, Milhorat and Bartels (115) have suggested that  $\alpha$ -tocopherol plus inositol or other hydroxylic agents may form curative mixtures which can accomplish what vitamin E is unable to do alone (114). We mention this indecisive material because it exemplifies our ignorance concerning the carrier, fixative agent, and enzyme constituents (steps 8, 9, and 10) of  $\alpha$ -tocopherol. Elucidation of the fundamental chemistry offers the best chance of curing these appalling diseases.

**Encephalomalacia and Exudative Diathesis in the Chick.**—Dam and his associates (26) have described two derangements which are separately



associated with vitamin E deficiency, each requiring a different specialized diet for development. While it is not possible to say that the diathesis is a primary or even uncomplicated vitamin E deficiency, it would appear that the encephalomalacia involves impairment of the essential tissue function and is thus a primary syndrome. Large areas of brain tissue fail to develop or are destroyed in the encephalic syndrome. The relative potencies of  $\alpha$ - and  $\gamma$ -tocopherols have not been tested in remedying this condition.

There is a certain similarity between the dystrophic, resorptive, and encephalomalacial syndromes. Each is caused by shortage of the same component part of tissue— $\alpha$ -tocopherol—without which tissue disintegrates or fails to be created.

## 2. *Secondary Vitamin E Functions*

Under this heading we have assembled a number of miscellaneous subjects and observations which, although scattered among the kitchen, laboratory, farm, and hospital, have these qualities in common—they are all connected with the E vitamins and the connection is indirect or multiple and does not concern tissue metabolism. The topics are grouped as follows: synergistic effects of tocopherols (1) as antioxidants in the economic and vital cycle, (2) as physiological antioxidants in general, (3) as observed in studies of human blood plasma, (4) on response to other vitamins and hormones, (5) on the lipide oxidation-reduction balance of the body, and (6) in reproduction.

**Synergy in the Economic and Vital Cycle.**—(a) *Quantitative Considerations.*—Much misunderstanding exists concerning the quantity of an antioxidant needed to protect a given quantity of carotene, vitamin A, and other labile substances. As far as we know, there is no fixed interrelation. The quantitative relationship is with the *medium*, not the substance protected. Every time a labile substance passes to a *new medium* it has a *new requirement* of antioxidant.

Thus a rich concentrate of vitamin A may require a 0.5% content of tocopherols to effect optimum stabilization. A dilute marine oil would need only 0.2% for protection. To prevent rancidity, purified cottonseed oil would take 0.08%; margarine, 0.045%; lard, 0.02%; etc. These figures are by way of example only, and are not accurate directions for stabilizing oils. Also, no account is taken of other synergists which could diminish the quantities of tocopherols required (112). A meal in a human stomach would need about 0.01% for optimum protection, while the whole human body, including water but not skeleton, apparently requires about 0.007% tocopherols. Consider, now, the possible fate of a certain small batch of 2 mg., or 5000 units, of natural vitamin A esters.

This would originate in a fish liver associated with oil containing about 0.03% tocopherols. Let the liver be rendered and the oil distilled to increase the concentration of vitamin A fifteenfold to a potency of 200,000 units per gram. The concentration of tocopherols will have been raised to 0.45%, which will effect admirable stabilization. Suppose, now, the concentrate is diluted 200 times with sardine oil to give a poultry oil containing a thousand units of vitamin A per gram. The mixture will contain 0.45% + 200 or 0.00225% of tocopherols (always supposing that the original sardine oil contained none), and it will be necessary to add nearly 100 times as much of tocopherols if it is desired to re-establish the initial margin of safety. Again, suppose that the fortified and stabilized sardine oil is added to poultry feed; a new tocopherol requirement is at once established, characteristic of the volume and components of the feed. Alternatively, let the 2 mg. of vitamin A find its way into 0.5 lb. of margarine. There it will become associated with half the concentration, but 100 times the absolute amount, of tocopherols, as in the fish-liver distillate. Consumed by a human at the rate of an ounce a day, the vitamin A will meet, in the adequately nourished stomach, more vitamin A from other sources and perhaps 1000 times more than the original vitamin E, also from other sources.

The two questions so frequently asked are: "How much vitamin E does it take to stabilize vitamin A?" and "How much to synergize vitamin A or carotene?" They can be answered by: "It depends on the medium," and "It depends on the animal." Each medium has its preferred concentration of antioxidant, each animal its proper quantity of tocopherols. It is a question of economics and ultimate destination whether a vehicle should be stabilized with vitamin E or a less expensive antioxidant. It would be economically unsound to feed vitamin E for the sole purpose of sparing the A vitamins. The quantity spared would cost many times more than its value. However, in any animal supplied with adequate quantities of vitamin E for general health, the vitamin A will be properly spared. Evidently the A vitamins are *indicators* which can be used to assess the dietary status with regard to vitamin E. However, if an edible vehicle such as salad oil or lard is stabilized with a non-physiological antioxidant, it may give adequate intestinal synergy, but it is not contributing its quota of vitamin E to the diet. Under such conditions, the survival of the A vitamins would give a false indication of vitamin E status. The whole question of whether a naturally unstable, edible fat shall be left in that condition or shall be stabilized for marketing and the convenience of the housewife, or whether it shall be fortified with vitamin E so as to contribute its proper quota to the diet, is an economic and health question that requires considerable "thinking through."

(b) *Qualitative Considerations.*—It is current practice to compare antioxidants with hydroquinone as though the various concentrations of tocopherol, of ascorbyl palmitate, of gum guaiac, and of hydroquinone, for

instance, that produce the same induction period in fat make them equivalent protective agents for that fat. Reference to the step diagrams of Figure 1 (page 472) shows how inadequate such an appraisal would be. Hydroquinone will preserve a fat excellently, but only between steps 2 and 5, for as soon as the fat mingles with aqueous media, the hydroquinone leaves it. Heavily substituted hydroquinones survive step 6, but are probably rejected by the gut wall, certainly by subsequent tissues. The same applies to gum guaiac or nordihydroguaiaretic acid (NDGA) (98). The furthest step to which ascorbyl palmitate (132) can travel has not yet been determined. Gossypol, which occurs plentifully in nature and commences its protection at step 0, with the genesis of the oil-soluble vitamins, must be removed before step 5 because it is poisonous (80). Beta- and  $\gamma$ -tocopherols exert their influence over the first eight or nine steps but  $\alpha$ -tocopherol, alone of all lipide synergists known, holds sway through the entire cycle of twelve steps. Of the naturally occurring water-soluble antioxidants, little is understood, but evidently ascorbic acid and *p*-aminobenzoic acid also exert their influence over much of the cycle. It is  $\alpha$ -tocopherol and ascorbic acid which determine chiefly the stability of the food in the field at the start of the journey, and the reducing power or stability of the blood at the finish.

**Physiological Antioxidant Phenomena.**—The power of vitamin E to repress oxidation in the intestinal tract was brought to light in studies on carotene and vitamin A conducted by Moore, Davies (29, 118) (liver storage), Sherman (138), Quackenbush, Cox, Steenbock (128) (utilization of carotene), and Harris, Hickman, and Kaley (67, 73) and others (147) (U.S.P. vitamin A assay method).

**Liver Storage.**—When an ample but controlled quantity of vitamin A in the form of the free alcohol or acetate or natural esters is fed to laboratory rats, the quantity found in the liver on autopsy increases according to the quantity and kind of vitamin E fed at the same time as vitamin A. All three tocopherols are equally effective (88). Too small or *too large* dosages of vitamin E show depressed effect and the vitamin E esters are scarcely active at all. Since esterification removes their immediate antioxidant property and the tocopherols are known to be hydrolyzed during diffusion through the intestinal wall into the circulation, the evidence points definitely to intestinal protection by the free tocopherols, especially when administered in quantity insufficient for the quinone fraction to exert a countereffect (see Table II). The failure of some workers to confirm all the results of Moore, and the writers' laboratory is attributed to lack of appreciation of the factors involved (8, 56). Nevertheless, in spite of the

TABLE II  
LIVER STORAGE OF VITAMIN A AS INFLUENCED BY TOCOPHEROLS

Treatment		Duration, weeks	Vitamin A storage						Ref. No.
Vitamin A, units/day	Vitamin E, mg./day pherol		-E			+E			
			Concen- tration, units/g. Liver	Total, units	Recov- ery, %	Concen- tration, units/g. liver	Total, units	Recov- ery, %	
143 as halibut oil	0.43 <i>dl</i> - $\alpha$ -toco- pherol	24	395	4,650	19	1,300	13,500	56	118
217 as cod-liver oil	0.9 <i>dl</i> - $\alpha$ -toco- pherol	8	308	2,570	19	383	3,365	27	8
200 (total) as vitamin A in oil	10 <i>dl</i> - $\alpha$ -toco- pherol	(4 days)		18.5	9		45.0	23	57
265 (total) as carotene in oil	10 <i>dl</i> - $\alpha$ -toco- pherol	(4 days)		4.5	2		18.0	7	
250 (total) as carotene in carrots	1.0 <i>dl</i> - $\alpha$ -toco- pherol	(4 days)		13.2	5		27.2	11	
70 as vitamin A pal- mitate	0.5 <i>d</i> - $\alpha$ -toco- pherol	2	75	302	31	101	498	51	64
	0.5 <i>d</i> - $\gamma$ -toco- pherol	2	75	302	31	91	479	49	
70 as U.S.P. Reference Oil 2	0.5 <i>d</i> - $\alpha$ -toco- pherol	2		157	16		292	34	64
None*	0.014 <i>dl</i> - $\alpha$ -toco- pherol	32		9			5,800		29

\* At start of experiment all rats had about 20,000 units of vitamin A stored in their liver.

generalizations just enumerated, it is probable that many minor factors affecting liver storage remain to be disclosed.

**Growth Responses.**—When weanling white rats are reared on the depletion diet used, for instance, for the U.S.P. method of vitamin A assay, the time of depletion is delayed when optimum quantities of tocopherols are fed, as shown in Figure 3.

Again, if suboptimal quantities of vitamin A alcohol or carotene are fed to groups of the depleted animals, together with vitamin E in quantities increasing from group to group, the growth promoted by the A vitamin is increased—~~very~~ greatly with the optimum quantity of vitamin E. The trend is shown in graphs reproduced from Hickman and associates (73) (Fig. 4); this positive synergy is produced equally by all three tocopherols, by such fat-soluble antioxidants as lauryl- and isoamylhydroquinone, and by ascorbyl palmitate. The greatest protection of vitamin A is given by mixtures of synergists, as would be anticipated from the work of Mattill (112). Table III displays some of the results.

TABLE III  
GROWTH RESPONSE OF VITAMIN A DEPLETED RATS TO MINIMAL DOSES OF VITAMIN A AND CAROTENE WITH AND WITHOUT SIMULTANEOUSLY ADMINISTERED ANTIOXIDANTS (74)\*

Vitamin fed daily	Amount, $\mu$ g.	Antioxidant or covitamin fed daily	Amount, mg.	Average gain in weight in 36 days, g.
Crystalline vitamin A	0.57	Control	..	30.1
		Mixed natural tocopherols†	0.13	57.7
		<i>d</i> - $\alpha$ -Tocopherol	0.15	48.9
		<i>d</i> - $\beta$ -Tocopherol	0.15	48.8
		<i>d</i> - $\gamma$ -Tocopherol	0.15	45.8
		<i>d</i> - $\alpha$ -Tocopheryl succinate	0.15	35.0
		Hydroquinone	0.15	30.8
		Laurylhydroquinone	0.15	54.5
		<i>p</i> -Aminobenzoic acid	0.15	30.8
Crystalline carotene	1.20	Control	..	21.6
		Mixed natural tocopherols†	0.25	63.1
		<i>dl</i> - $\alpha$ -Tocopheryl acetate	0.30	24.3
		Ascorbyl palmitate	1.00	50.5
		Ascorbyl palmitate plus mixed natural tocopherols	1.00/0.25/	80.8

\* Ten rats per group.

† From molecular distillation of mixed cottonseed and soybean oils.

**Vitamin A Bioassay.**—Nature—to endow her with wisdom—has taken some cognizance of the synergy and supplied most of her provitamin A accompanied by tocopherols, ascorbic acid, and phenolic co-stabilizers. In the green pea, for instance, the Emmerie-Engel value for the crude lipide extract is nearly six times greater than accounted for by the toco-

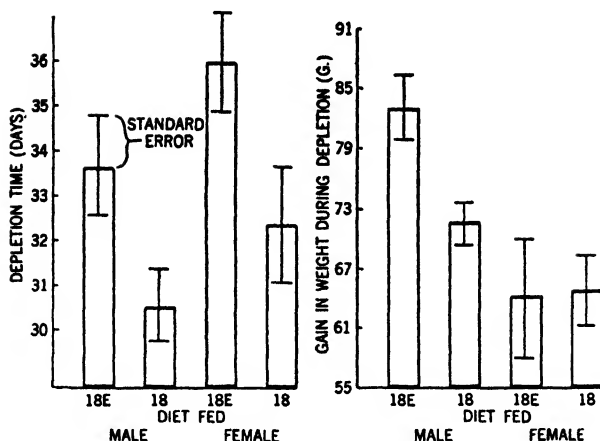


Fig. 3.—Effect of tocopherols on depletion time and weight gain of rats. Diets 18 and 18E were identical (U.S.P. Vitamin A-free ration containing olive oil) except that diet 18E contained natural mixed tocopherols to supply 0.5 mg. tocopherols per 10 g. of diet.

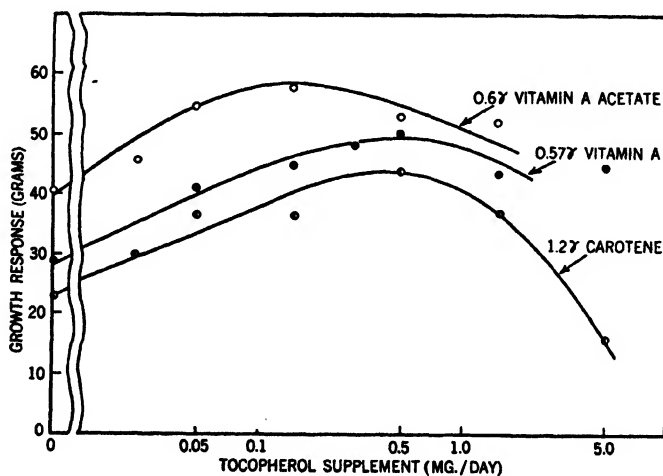


Fig. 4.—Sparing activity of tocopherol on minimal quantities of vitamin A, vitamin A acetate, and carotene. Gain in weight of vitamin A-depleted rats in 28-day period reaches an optimum with 0.1 to 0.5 mg. tocopherols daily.

pherols. Vitamin A in meat is stabilized by less vitamin E and by the B vitamins, including *p*-aminobenzoic acid, pyridoxine, etc. Marine oils alone present their vitamin A inadequately stabilized for absorption through the animal gastrointestinal tract, though stabilization media from other sources are generally present in the tract. Under conditions of nutrition heretofore considered favorable to man, the natural protection of the A vitamins may be adequate, but it is certainly not optimum, since ingestion of extra vitamin E sharply increases the survival of vitamin A, for instance in human feces, as shown in Table IV.

TABLE IV  
EFFECT OF  $\alpha$ -TOCOPHEROL ON FECAL EXCRETION OF CAROTENE IN HUMANS (74)  
AND IN RATS (57)

Tocopherol fed, mg.	a	Apparent carotene recovered			
		b	c	(c - b)	$\frac{(c - b)}{a} \times 100$
	Carotene fed, mg.	Gross, mg.		Net, mg.	Proportion of that fed, %
A. HUMANS					
0	0	5.1	10.7	5.6-6.2	28-31
0	20.0				
0	0	4.5			
0	0	4.5	15.0	9.9-11.5	50-58
100	20.0				
0	0	4.7			
B. RATS					
0	0.265 in oil	0.030			19
0.1		0.038			24
0.5		0.047			29
2.0		0.055			36
10.0		0.066			41
0	0.250 in carrots	0.068			45
0.05		0.081			54
0.2		0.083			55
0.5		0.086			57
1.0		0.104			69

The conditions in the U.S.P. bioassay are far different from those of human or farm-animal diet. All the constituents of the assay diet, except one, are virtually devoid of vitamin E. The exception, the specified "5.0% of vegetable fat" may contribute antagonists, sparing agents, or nothing to the better utilization of vitamin A, as suggested in Table V. In the protracted early attempts to balance the  $\beta$ -carotene international standard

with the practical fish oil substandards, the diluent oils (coconut, sesame, and olive) were low in tocopherol, and supplies earmarked for long-term assay progressed, we fear, from incipient to marked rancidity with the

TABLE V  
EFFECT OF DIETARY FAT ON UTILIZATION OF MINIMAL QUANTITIES OF VITAMIN A OR CAROTENE

Fat used		Tocopherol content, %	Approx. modification of A potency
Name	Properties		
Coconut	Neutral	0	Unity
Olive	Oxidative	0	-50%
Cottonseed	Slightly protective	0.08 (alpha)	+50%
Soybean	Highly protective	0.16 (gamma)	+75%

passage of time. Hence, the tremendous interlaboratory variation of growth from unit intake of vitamin A. Today the diluent, which in America is, by almost common consent, freshly opened cottonseed oil, provides a nearly perfect medium; and current anxiety is for the Reference Oil U.S.P. Nos. 2 and 3, which show signs of gross instability (19).

The implications of the A-E synergy do not cease with the provision of a suitable assay medium. Humans and farm animals do not receive dietary fat in the form of fresh cottonseed oil. Butter, lard, mutton, and beef tallow low in vitamin E, and shortening partially rancidified in stale pies and pastries contribute most of the fat of our diet, which is very much lower than the best U.S.P. assay diet in vitamin E. A vitamin preparation which yielded an entirely satisfactory assay in the laboratory might fail as a component in human or farm-animal diet; for instance, distilled vitamin A esters and crudely saponified fish-liver oils would compare well on a high E diet, but the esters, alone, would measure up on low vitamin E. Similarly, vitamin A acetate in oil,  $\beta$ -carotene in butter, and vitamin A in soup made from a dehydrated powder would all assay well with a diet containing cottonseed oil, but the butter and soup would receive a low rating in a diet containing olive oil.

For a critical evaluation of a vitamin A product we believe the assay should be conducted twice, once with the 5.0% of specified fat, ascertained to be scrupulously fresh and reinforced with so much vitamin E that both vitamin A and carotenoids are utilized to their maximum. The second assay should be done simultaneously with equally fresh nonrancid fat, such as lard or olive oil, which contains no vitamin E. Both assays will be done matching the unknown with a standard reference oil—preferably vitamin A acetate in stabilized vegetable oil. (Discussions concerning an improved national standard for vitamin A are now taking place.) When the unknown shows the *same* relative match with the standard in both assays, the unknown will be considered to have



good inherent stability. When it is lower in the second assay, the instability will be proportional to the difference in ratings.

*Synergy with Essential Fatty Acids.*—The tocopherols exert an anomalous sparing action on the essential fatty acids. Rats deprived of such foods as linoleic, linolenic, and arachidonic develop a syndrome, described by Burr and Barnes (15), which includes scaliness of the tail, sore feet, and uncouthness of the hair. At the same time, the water balance of the body is displaced. The symptoms can be relieved in degrees, the paw condition being the first to clear up, then the tail, and finally the general appearance of well-being is resumed. Ordinarily about 20 mg. daily will form the minimum prophylactic dose; 40 mg. will be curative. Suppose, now, the animals are given *half* the quantity of essential acid—one group without vitamin E, the other with a daily addition of 0.5 mg. *d*- $\alpha$ -tocopherol. Those with the vitamin E make a complete cure, those without it show little improvement over animals receiving no linoleate (81a). The vitamin E has made a subminimal quantity of linoleate into an adequate quantity. But here is the anomalous fact—rats receiving the depletion diet and *no* linoleate develop *worse* symptoms when given vitamin E. It is not difficult to provide at least a plausible hypothesis in explanation of this curious phenomenon. In the intestines, before absorption, the tocopherol saves the unstable fat from oxidation. After absorption, the essential fat passes to one site of operation, the tocopherol, chiefly but not completely, to others. It must be assumed that the function of the essential unsaturated fat is to promote oxidation, which it will do even in the presence of a *little* tocopherol. When, however, there is *no* dietary essential fat and the animal is existing in ill health on stored material or traces of unsaturated fat manufactured endogenously, administration of tocopherol will so lower the oxidative capacity of these traces that the deficiency symptoms increase.

*Protective Action on the Stomach Itself.*—During an investigation on chronic, induced alcoholism in rats, it was found that animals maintained on diets which were low in any of the following factors—vitamin A, essential fatty acids, and pyridoxine—developed, more often than not, large ulcers of the stomach. In every case examined, the animals developed no ulcers if they received a supplement of 0.5 mg. of tocopherols daily (68, 87).

Any or all of three effects may contribute to the preventive action of the vitamin E. The other vitamins may be spared to a point where they are sufficient to maintain epithelial integrity. Peroxides may be destroyed, which would otherwise serve as tissue irritants. Finally,  $\alpha$ -tocopherol may be necessary for the repair of stomach tissue, just as it is necessary for the manufacture of any tissue. It is not known, at present, whether this

development (and its prevention by vitamin E) of stomach ulcers is peculiar to the rat under the abnormal conditions of the assay laboratory or whether this is a lead which may be fruitful in clinical medicine, but it is clearly worth following.

*Special Case of the Dairy Cow.*—The fat of an animal—rat, pig, or cow, for instance—fed tocopherols resists rancidity in proportion to the quantity fed (Table VI). As a broad concept, we may consider the tocopherols to extend protection from oxidation from the stomach to the whole organism; with adequate vitamin E, an animal is less likely to be oxidized.

TABLE VI  
INCREASED STABILITY OF BODY FAT RESULTING FROM TOCOPHEROL INGESTION (61, 69)

Species	Compound fed	Amount, mg.	Feeding period	Induction period		
				By O <sub>2</sub> absorption at 100° C., min.	By peroxide accumulation at 63° C., days	By peroxide accumulation at 100° C., hrs.
Rat	None (control)	...	Single dose	31	1.5	
	<i>dl</i> - $\alpha$ -Tocopherol	50		418	21.0	
	<i>dl</i> - $\gamma$ -Tocopherol	50		211	13.0	
Rat	None (control)	...	35 days			2.6
	<i>d</i> - $\alpha$ -Tocopherol	0.05				5.4
		0.5				6.8
		5.0				13.2
Pig	None (control)	...	30 weeks			4.9
	Natural mixed tocopherols	1.5/kilo body wt.				6.0
		15.0/kilo body wt.				10.0

Of all animals, the highly bred dairy cow would seem particularly destined to benefit from extra vitamin E. Her enhanced capacity for giving milk means that both fat- and water-soluble vitamins are being washed out at an increased rate. Her subsistence for half the year on stored and partially decomposed feed (always supposing that the fresh feed would have been adequate?) means that supplies of essential nutrients are curtailed. Not only have the vitamins diminished, but antagonists and antivitamin may have been formed. As a result, the color, quality, and quantity of the milk and butterfat fluctuate seasonally; the animal is a prey to infection, mastitis, and breeding irregularities, which latter include likelihood of abortion, and difficulties in freshening.

Silage is low in vitamin C, almost devoid of vitamin E. Yet vitamins E and C together account for the greater part of the reducing value of the blood, lymph, and fat. Increasing vitamin E should increase the yield of

A vitamin from the silage, restore the reducing power of the lipide fraction of the cow's blood plasma, and provide  $\alpha$ -tocopherol for the stabilization of milk fat and production of the fetus during pregnancy.

Previous experiments on vitamin E in the cow have suffered from one or all of the following mistakes: too few animals were used for too little time; the vitamin E was given with too much oil, as in wheat-germ oil, in the wrong dose or in unsuitable form (esterified); or the animals were not properly supervised.

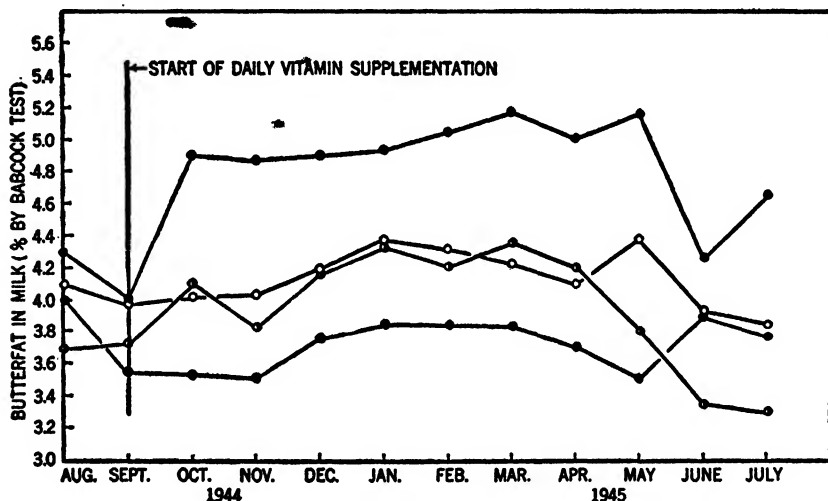


Fig. 5.—Effect of vitamin supplementation on the butterfat concentration in the milk of dairy cows: ○-○ represents average values obtained from group receiving 250,000 units vitamin A daily; ●-●, group receiving 1.0 g. natural mixed tocopherols daily; ◐-◐, group receiving both vitamins A and E; ○-◐, control group receiving no vitamin supplements.

The writers' laboratory conducted experiments for several years on various farms with contradictory results. The current experiment has embraced 40 cows, in 4 groups of 10, over a period of a year, and will be continued for at least another year. Our representative feeds the supplement to each individual cow daily, weighs the milk, and collects samples himself for analysis. The supplement is in the form of distilled, mixed natural tocopherols (1 g. dispersed in 25 g. of soybean flour) which is laid on top of the cow's feed in the manger. The effect on butterfat, in comparison with groups receiving, respectively, no supplement, vitamin A, and vitamins A and E, is shown in Figure 5. The report of an independent veterinarian who was uninformed concerning the experiment is shown in Tables VII and VIII, which also record milk quality and data concerning vitamin content (70).

TABLE VII  
INFLUENCE OF VITAMIN SUPPLEMENTS ON MILK AND BUTTERFAT PRODUCTION AND VITAMIN CONTENT OF MILK  
OF DAIRY COWS (70)\*

Factor	Supplement (daily)†	Time (days)					
		0	30	60	90	120	150
Milk production	Control, lbs. per day	29.2	26.2	25.3	24.1	22.4	20.6
	Vitamin A { % variation from value of control group	+4	-5	-5	-8	-13	-17
Butterfat concentration	Control, % in milk	3.73	4.10	3.83	4.17	4.33	4.20
	Vitamin A { % variation from value of control group	-4	-14	-9	-10	-11	-8
Total fat output	Control, lbs. per day	1.09	1.07	0.97	1.01	0.97	0.86
	Vitamin A { % variation from value of control group	+7	-2	+5	+0	+1	+3
Vitamin A output	Control, units per day in thousands	15.1	18.5	15.6	16.0	15.4	13.7
	Vitamin A { % variation from value of control group	-2	+55	+80	+130	+105	+85
Carotene output	Control, units per day in thousands	10.0	11.9	9.5	9.1	6.8	5.6
	Vitamin A { % variation from value of control group	+8	-30	-23	-18	-33	-33
	Vitamin E	-17	-20	+5	+2	+0	+0

\* Five to eight cows per group. Data from only those cows who lactated throughout this five-month test period were included in the averages calculated for this table. Production records for those animals who dried up or freshened in this period were omitted. The full detailed data will be published later.

† The control group received no vitamin supplement. The vitamin A group was fed 250,000 units of vitamin A. The vitamin E group was fed 1.0 g. natural mixed tocopherol. The group receiving vitamins A + E was fed 250,000 units of vitamin A plus 1.0 g. mixed tocopherol daily.

There are too many implications in this cow experiment to discuss here. There is the influence of vitamin E on the balance between carotene and vitamin A; and there are the peculiar effects of vitamin A when administered in quantity to the cow, alone or with vitamin E. All of these phenomena will be subjects of a separate article, but some of them will be referred to in later sections of this article.

TABLE VIII  
EFFECT OF TOCOPHEROL SUPPLEMENT ON GENERAL HEALTH AND REPRODUCTIVE  
PERFORMANCE OF DAIRY COWS (70)\*

Year	Vitamin supplement (daily)	General health and appearance	Appetite	Incidence of infections	Ratio of services to conceptions	Remarks
1943	None	Fair	Poor	Some, but not severe	5 to 1	Four abortions and three stillbirths. Retained placenta, a common fault necessitating the services of a veterinarian after each parturition
1944	1.0 g. natural mixed tocopherols	Much improved	Excellent	Less mastitis. No generalized herd infection	3 to 1	One abortion and no stillbirths. Veterinarian's services required only once during year (retained fetal membrane)

\* Veterinarian's report: A group of 20 cows, a portion of a herd of purebred Brown Swiss dairy cows.

**Synergies as Observed in Human Blood Plasma.**—Perhaps the greatest hiatus in present-day vitamin investigation occurs between laboratory studies on animals and applied human medicine. The human patient is only occasionally available for drastic experiment and, generally speaking, depletions must be spontaneous, medication should be conservative, and the biopsy specimen is limited to blood and urine. Studies on human plasma thus have a very special significance in connection with the vitamins. If there are synergies, one vitamin protecting or wasting another, these would be expected to show as changes in vitamin concentration in the plasma. Particularly, if the tocopherols are protective for the A

vitamins, vitamins C and D, and the hormones, higher plasma concentrations of these entities should follow tocopherol medication. Again, plasma contains the lipides, proteins, or other factors which convey the tocopherols. Studies of whole and fractionated plasma offer a route to understanding both primary and secondary tocopherol functions. This must be the excuse for a digression to some clinical experiments that do not, at first sight, appear connected with the main theme.

*Plasma Variations in an Individual.*—The object of the experiment was to find out how doses of individual vitamins A, E, C and carotene affect the concentrations of A, E, C, and carotene in human plasma.

In order to learn the factors likely to be encountered in blood plasma surveys of large groups, analyses were done weekly (or more often) on blood taken from the two writers. Samples (30 ml.) were oxalated, centrifuged, and examined for carotene (91), tocopherols (129), and vitamins A (91) and C (10, 116), the latter within half an hour of withdrawal. When the general trends had been ascertained, large single, or smaller continued, doses of one of the vitamins were swallowed and the effect noted on the others. A massive dose of vitamin A (0.5 million units) raised the plasma level of vitamin A from our normal 200 units per 100 ml. to 750 units for a few days, after which the level returned to 10% above normal. A day after administration of the A, the level of vitamin C dropped 20% for a few days. Increasing the intake of vitamin E had no perceptible effect on the level of vitamin A. Extra vitamin C was substantially without effect on the levels of vitamins A and E.

The trends in levels were followed by plotting the log concentrations against time. The curves were thus all exactly comparable, irrespective of units chosen. It was soon apparent that there were long-term seasonal variations in level which affected all four vitamins and were *not directly connected with vitamin intake*. Interruption of our normal routine by a journey lasting six weeks caused the curves for one of us to become abruptly discontinuous with previous trends. Yet the food intake (eggs for breakfast, meat for dinner, etc.) remained fairly similar. *Changed habit of life was more effective than vitamin supplements in changing the plasma levels of these vitamins.*

*Plasma Variations in a Group of Persons.*—Following this experience, a carefully controlled experiment was arranged by Dr. C. Urbach on conscientious objectors available to the Children's Hospital of Philadelphia. Plasma levels of vitamins A, C, E, and carotene were followed in five groups during a preliminary period of some months. One group was continued as a control and of the others, each one received massive doses of one of the vitamins. After another six months, when war circumstances called a halt to the experiments, the plasma levels had become adjusted as shown in Table IX.

TABLE IX  
SPONTANEOUS CHANGES IN HUMAN BLOOD VITAMIN LEVELS COMPARED WITH CHANGES INDUCED BY VITAMIN SUPPLEMENTATION (153)

Group	Extra vitamin ingested			Average plasma alteration of groups			
	Daily dose first month	Daily dose second 5 months	Per cent of Natl. Res. Council recommendations	Carotene	Vitamin A	Vitamin E	Vitamin C
Control	None	None	None	None*	None*	None*	None*
Carotene	30.4 mg.	15.5 mg.	1,000-520	+150%	None	-5%	+10%
Vitamin A (as natural esters)	14.8 mg. (51,800 units)	13.3 mg.	1,000-930	None	+30% diminishing to +10%	None	±10%
Tocopherols (mixed natural, approx. 50% α- and 50% γ-)	91.7 mg.	60.0 mg.	370-240†	+30%	None	+20%	None
Vitamin C	160.0 mg.	116.3 mg.	215-155	None	None	None	+50%
Spontaneous variation above minimum plasma values:							
Composite average of 5 groups, %				105	50	80	90
Smallest and largest individual variation <i>not</i> receiving tabulated vitamins, %				89-330	56-264	150-323	175-10,000
Smallest and largest individual variation <i>receiving</i> tabulated vitamins, %				51-111	60-110	67-171	55-110

\* The values for the controls are the basis for calculating the alteration of the others.

† Requirements estimated at 25 mg. per day. Average food intake believed to contain 15 mg. per day.

Note how small the plasma alterations are, compared with the dosage. The two "short period" supplements (having half-periods of body adjustment of a few weeks), carotene and vitamin C, caused an increase of 150% and 50%, respectively, which represented less than 35% plasma response to the extra dosage. Months of massive dosage with vitamin A caused less than 30% plasma alteration, corresponding with 3% adjustment to changed intake. Compare these figures with the *spontaneous* group-average seasonal variations of all the groups, which range from 50% for vitamin A (that is to say, spontaneous variations were nearly twice as great as caused by 1000% alteration of intake) to 105% for carotene; or the spontaneous variations for single individuals, ranging from 56–264% for vitamin A to 175–10,000% for vitamin C, where these vitamins were not given, and 60–110% (vitamin A) and 55–110% (vitamin C), when they were given. While many minor deductions can be made from these data, one major point demands recognition; namely, that there are factors unconnected with vitamin intake which affect, profoundly, the quantities of vitamin carried by plasma. The figures quoted are not just the usual uncertainties of bioassay to be averaged away by statistical analysis; they are records of individual events which cry for detailed explanation.

All through these experiments careful records of diets were kept, so that the variation in vitamin intake could be calculated. It was soon apparent that the variations from day to day and from one individual to another were trivial in comparison with the quantities used in medication, which, in turn, elicited so little plasma response (153).

*High Doses of Vitamin A.*—In his experiments, variously on hyperkeratosis, maternity health, and tumor remission, Straumfjord has given patients 100,000 U.S.P. units of vitamin A daily, for periods of months and years (144, 145). This quantity, which is twenty times the recognized daily dose, has produced permanent increases in the plasma of only 65%, on the average.

*Experiments at the Hillman Nutrition Clinic.*—The measurements outlined above were all made on persons receiving *more* than the usual amount of vitamins. The underprivileged patients attending the Alabama Hospital have proved an excellent source of subjects receiving less than normal vitamin intake.

Analyses of blood plasma for vitamins A, C, E, and carotene on nearly 300 patients have shown a curious uniformity of displacement of values. The underprivileged, like the normals, show both low and high plasma levels, according to person and circumstance, but the distribution curves of the two groups are widely separated and, with the



exception of vitamin C, are all separated by about the same amount (see Fig. 6). The underprivileged are 30% lower than the normals. Stated in reverse, the normals are 43% higher. Now, according to the Urbach and Straumfjord experiments, the normals would have to receive ten or twenty times as much vitamin A, five to ten times as much vitamin E, but only one-half as much again of carotene as the depressed group, to cause these differences. Even in the saddest circumstances of the South, this gross distortion of dietary intake is unlikely. It is far more likely that the whole acceptor and carrier system for the lipide-soluble vitamins is deranged in the underprivileged.

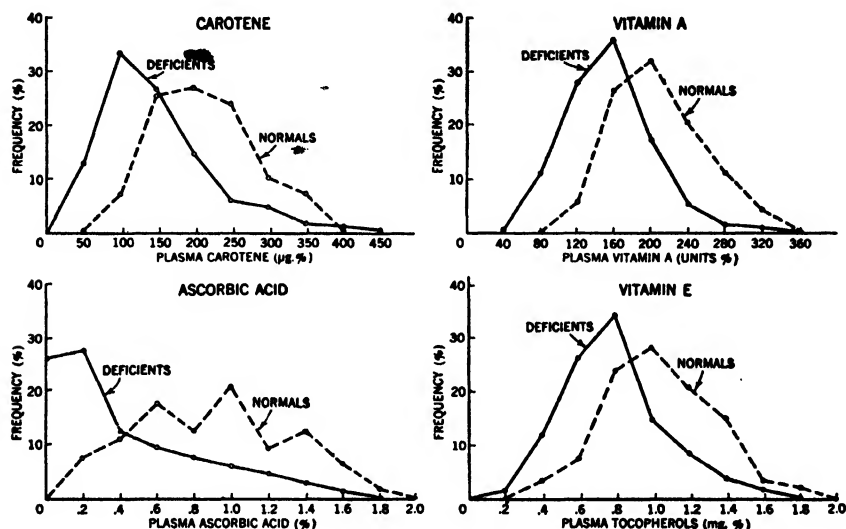


Fig. 6.—Distribution curves showing the blood vitamin levels of "deficients" (persons of low income in a region where malnutrition is endemic) and of "normals" (persons in good health in the same region) obtained at the Hillman Nutrition Clinic (63).

Examples of discrepancy between intake and response can be drawn from many fields of nutrition. Thus, Borsook was able to point only to trivial objective change in factory employees supplemented with double the normal intake of vitamins for long periods (14). Writing of human subjects deprived of all sources of vitamin A, the British Vitamin A Subcommittee of the Accessory Food Factors Committee (86) confesses:

"The value of the final rod threshold in all subjects, whether undergoing depletion or not, deteriorated significantly during the first winter, seven months from the start, but in most cases recovered in the spring to the initial level. The explanation of this phenomenon is unknown but the risk of misinterpretation thus introduced is clearly great."

Again, in a fish hatchery report in 1942 (151), we find the statement that the wild brook trout contains much vitamin A in its liver. Trout bred in captivity have little vitamin A and the quantity in the liver is not altered by feeding carotene, butter, or cod liver oil, but is increased when beef liver is added to the diet. It could be inferred that the beef liver supplies the carriers or fixatives to enable the trout to absorb and retain the A vitamins.

*Vitamin Interrelationships in Plasma.*—Referring to the Philadelphia experiment again, the protection of A, E, and carotene expected from extra vitamin C was conspicuous by its absence. The depression of carotene expected from vitamin A was not observed but may have been present, since the method of analysis measures only yellow color. The color of the carotene may have been replaced by metabolites of vitamin A. For example, rats fed much vitamin A turn distinctly yellow. Feeding carotene definitely increases the ascorbic acid in the plasma; extra vitamin E preserves the carotene. Bessey and Lowry, examining their records on the public school children of New York City, also find a positive correlation between carotene plasma values and vitamin C (11).

*Response to Other Vitamins and Hormones.*—Our general hypothesis would predict that tissue and fat which have been stabilized against spontaneous oxidation should extend the life of other labile substances of exogenous or endogenous origin residing therein. The animal body presents enormous interfacial areas between lipide and aqueous media, so that the question of solubility need not intrude.

*Vitamin D.*—Over the range of tocopherol intake considered normal (0.2–2.0 mg. per day) for the laboratory rat, there is no discernible influence on the "line test" for vitamins D<sub>2</sub> and D<sub>3</sub> (66). Large doses of 10–100 times the normal intake increase the requirements of vitamin D. Weissberger and Harris have followed the phosphorus turnover in the skeleton of the rat with varying doses of vitamin E (165). The turnover is raised on a deficient diet or one containing 100 mg. per day, but remains entirely normal over the range 0.1–10.0 mg. per day. The interplay between tocopherols and calcification may be observed in the repression of tartar formation on human teeth (see below). It should be noted that the new "antistiffness" factor of Wulzen and van Wagtendonk also influences calcium deposition (154).

*Hormones.*—Work that was interrupted by the war suggested that  $\alpha$ -tocopherol spares progesterone (140) and testosterone (1). Our own attempts to extend the activity of testosterone by vitamin E in the castrated cockerel met with failure, not because the synergy was unobserved,

but because as many birds showed reduction in comb growth as showed extra growth—the experiments were statistically worthless. Other experiments with ovariectomized rats and mice showed a definite estrogen-sparing potency for tocopherol in some trials, but statistically insignificant responses in other trials. The early work with animals requires careful repetition. At present, the best evidence for synergy between vitamin E and hormones rests on a few clinical reports (7, 141).

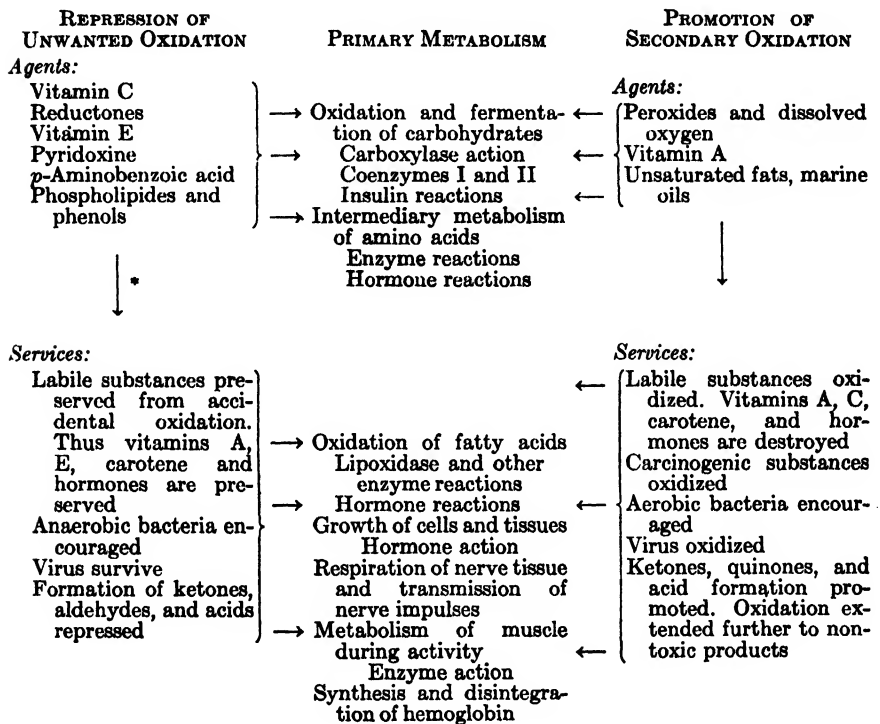
**Vitamin C.**—We have been able to observe no extending action of tocopherol on ascorbic acid, although the reverse reaction, in which ascorbic acid or ascorbyl palmitate extends  $\alpha$ -tocopherol, is readily observed. The effect of vitamin E on C was examined in the guinea pig because of its sensitiveness to lack of ascorbic acid. The tocopherols were unable to render a subminimal quantity of ascorbic acid protective against scurvy, nor did they increase the storage of ascorbic acid in the tissues.

**Lipide Oxidation-Reduction Balance in the Animal Body.**—Vitamin E, unsaturated fats, vitamin A, fatty liver, anoxia, starvation, carcinogens, longevity, cancer—through this heterogeneous collection, we shall try to chart a single theme: *the effect of covitamin E in maintaining oxidative normality*. In this connection, the body has three broad tasks: (1) to prevent *spontaneous* oxidation in a living vessel filled with easily oxidizable substances, surrounded and permeated by a reactive atmosphere; (2) to promote at will, through enzyme reactions, *wanted* oxidations (in movement, digestion, vision, and so forth); (3) to dispose by oxidation of those waste products and toxins which are not metabolized in the main enzyme cycles.

Task (1) is evidently fulfilled by the tocopherols and the other natural antioxidants. Examples of substances undertaking task (2) are the B vitamins and insulin. Task (3) is performed by the unsaturated fatty acids and, perhaps, vitamin A. The carcinogens, including butter yellow, would be examples of toxic products disposed of by peroxidizing fats.

If we accept for a minute this general picture, the body, in carrying out task (1) to best advantage, would seek enough  $\alpha$ -tocopherol to stabilize its muscle tissue and would augment this with an excess of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols and the water-soluble antioxidants to preserve the *status quo* for as long as possible. [McCay finds that addition of extra  $\alpha$ -tocopherol to the diet of rats on longevity experiments increases both the life span and the period of spermatogenic activity (104).] This, however, would tend to repress activity under task (2) and would leave the body loaded with unoxidized residues and poisons. A quota of peroxidizing fat would, therefore, be taken on and there would be established an equilibrium which,

under optimum conditions of nutrition, would enable all three tasks to be accomplished with minimum mutual interference. An equilibrium of this kind is suggested in Scheme 1.



SCHEME 1

PROOXIDANTS AND ANTIOXIDANTS IN BODY METABOLISM

An example of such an interplay and its specificity is shown by an *in vitro* experiment. A saturated solution of carotene in fresh olive oil is poured into test tubes. A milligram of vitamin A is added to one tube, a milligram of vitamin E to another, both A and E to a third, while no addition is made to a fourth. The tubes are aerated in the Swift apparatus at room temperature. Tube No. 4 bleaches in a few days, No. 3 takes a week, No. 1 fades in a few hours, and No. 2 takes a week. If estrone is substituted for the carotene in the olive oil, vitamin A lengthens the life of the aerated estrone and the estrone proves as good an antioxidant as tocopherol for the vitamin A.

\* Vertical arrows are signposts. Horizontal arrows represent opposing tendencies of forces.

So, in the same medium and at the same time, vitamin A can act as a prooxidant for carotene or as an antioxidant for estrone. Similarly, *in vivo*, the *proper* quantity of tocopherol can protect tissue from disintegration and vitamin A and carotene from oxidation, while still permitting unsaturated fat to detoxify butter yellow in the intestines. Shifting the field for a moment, we find György (59) causing acute cirrhosis in rats by feeding a high sucrose diet and a *little* unsaturated fat. He is unable to prevent the condition by adding tocopherol or more unsaturated fat to the diet *singly*, but obtains satisfactory protection when both are added together. Again, the carcinogen, 3,4-benzopyrene, aerated *in vitro* in oil solution, can be oxidized by unsaturated fats, while the oxidation of the fats is delayed, in turn, by the carcinogen (29a). Vitamin E delays the oxidation of both in the mixture.

TABLE X

EFFECT OF TOCOPHEROLS ON SURVIVAL TIMES OF ADULT MALE RATS SUBJECTED TO LOW ATMOSPHERIC PRESSURE (185 MM. HG AT 26° C.)

Diet*	Tocopherol supplement, mg.	Number of trials	Average survival time, min.	Average deviation from controls, %
Low in fat and vitamin E	0	8	34.4	...
	3.0	8	107.0	+211
5% lard, low in vitamin E	0	10	13.3	...
	0.5	10	31.4	+136
12% lard, low in vitamin E	0	3	14.0	...
	0.3	3	24.9	+78

\* The rats were given these diets and supplements for 15 to 20 days before decompression. They had previously been receiving commercial dog chow.

*Anoxia, Starvation, and Cancer.*—There are conditions under which it would be advantageous to disturb the balance illustrated in Scheme 1. Thus, in high altitude flight, the oxygen requirements could be reduced temporarily to a point where only the operational minimum of the task (2) reactions would be allowed to proceed. That manipulation of diet can promote this condition has been known since Campbell discovered that rats fed on carrots could withstand lower atmospheric pressures than usual (20). Under the stimulus of this experiment, many others have been published by other workers who have established that vitamin C, together with a carbohydrate diet, will give temporary resistance to anoxic anoxia (92). Recent work in our laboratory with white rats shows that vitamin E has an even greater effect in extending resistance to high altitudes (82)—see Table X.

The effect of vitamin E in conserving fat and tissue, and thus, the span of life under conditions of starvation or extreme malnutrition, is the subject of experiments now in progress.

The possible connection between the E vitamins and cancer has begun to interest a number of workers (21, 119). The cancer cell has an altered metabolism and oxygen quotient (160); vitamin E controls cell metabolism and adjusts the oxygen quotient (77). The incidence of cancer in mice is shifted according to the kind and quantity of fat in the diet (149); vitamin E controls the behavior of fat (111). Experimental cancers are induced by carcinogens; vitamin E and unsaturated fats together determine the fate of carcinogens (59). These three aspects have been given exploratory examination, but much more critical work remains to be done. Thus, while the  $\alpha$ -tocopherol content of normal tissue has been measured many times, we find no analyses of cancer tissue reported. The experiments of Houchin and Mattill have not been repeated with cancer tissue.

**Vitamin E in Pregnancy.**—Any theory of the utility of vitamin E in maternity nutrition must be elastic enough to accommodate the failures without damaging the main thesis. Our theory, retained in substantially the present form for the past three years, is as follows. To insure conception, the vitamin E status of both parents (integrity of the seminiferous epithelium of the male, etc.) must be assured. At conception, the female, who has undertaken the production of 7-9 lbs. of tissue in as many months, will require extra vitamin E to provide the " $\alpha$ -tocopherol-of-constitution." While this would not cause undue drain on dietary resources as a primary gain in weight of the parent, the secondary gain in weight for a separate fetus demands that the extra  $\alpha$ -tocopherol be transported across the placenta. A placental transfer factor,  $f_{so}$ , must be added to the others making up  $\psi$  for vitamin E. It will be seen from Table XI that  $f_{so}$  may be quite small, 20-50%. In the first few weeks of pregnancy, the *corpus luteum* hormone, progesterone, is produced by the mother to insure the attachment of the fetus to the uterus. If the hormone is produced in borderline or mildly deficient quantities, then ingestion of enough vitamin E can make it sufficient. At this time the mother's appetite may be enhanced, normal, or impaired. In the latter two events, benefit will accrue from the increased yields of such factors as carotene, vitamin A, and essential fats which vitamin E will deliver to the fetus. For the adult human we speak of a "maintaining dose" of a vitamin, but for the fetus we must provide a dose sufficient for *manufacture, maintenance, and endowment*. The child must be created and launched in life with sufficient reserve to tide over the limited dietary resources of the first few months. As though it consciously appreciated this situation, the body of the mother

undergoes changes which enrich the blood with tocopherols a few weeks before parturition.

Straumfjord and Quaife (146) have examined the blood of various groups of women and of mothers and children at birth (umbilical cord), and find the trends shown in Table XI. It has not yet been de-

TABLE XI

EFFECT OF PREGNANCY AND PARTURITION ON PLASMA VITAMIN LEVELS OF HUMANS (146)

Condition of subjects	Quantities of vitamins in blood (per 100 ml. plasma)			
	Vitamin A, units	Carotene, μg.	Vitamin C, mg.	Vitamin E, mg.
Nonpregnant women (controls)	188	140	0.8	1.20
Pregnant women (third trimester)	198	160	0.7	1.40
At parturition: women	182*	180	0.5	1.80
	130	20	1.2	0.35
	96			
Infant values as per cent of maternal values	average... 47 range..... 20-140	11 3-40	240 100-1000	19 6-65

\* After medication for some months with 50,000 to 100,000 units of vitamin A daily.

cided whether the low value of vitamin E in the plasma of the newborn reflects: (a) normal tissue contents associated with an abnormal blood composition; (b) faulty transfer across the placenta; or (c) efficient placental transfer followed by rapid fixation by the fetus. We speculate that the high maternity concentration of vitamin E and the abnormally high vitamin C in the child, at time of birth, are both measures undertaken in partial compensation for the low fetal vitamin E. It will be instructive to examine the blood of children from mothers who have received liberal supplements of vitamin E.

To summarize, the expectancy of response varies with circumstances. If the mother is in a borderline condition of health and nutriture, and if the endogenous supply of progesterone is suboptimal, vitamin E supplementation may make just the difference between abortion and a healthy child. In any event, ingestion of plenty of vitamin E will favor adequate reserve in the child and will increase both the fat and the oil-soluble vitamins in the mother's milk, again benefiting the child. If, however, health and nutriture are such that these factors are all taken care of by the usual diet, supplements of vitamin E during pregnancy will make no readily discernible difference. Again, if there are adverse factors in pregnancy (such as disease or mechanical obstruction) entirely unconnected with

nutrition or hormone balance, supplements of vitamin E will be powerless to avert disaster.

**Pigment Effects.**—Two pigments that can occur in the animal body and have been studied especially in the rat are controlled by the vitamin E balance. One normally occurring in the teeth disappears when the diet is low in tocopherols; the other, which is not present in the fully nourished animal, appears, noticeably in the uterus, after some months on a diet deficient in vitamin E (109). In the classification adopted in this chapter, we are at loss to know whether these should be placed with the primary or secondary E syndromes, but have chosen the latter, believing that the pigmentation of the uterus, at least, is due to abnormal oxidation of lipide followed by polymerization to a tarry substance. The dental phenomenon was first noticed by Davies and Moore (29) and has recently been re-examined by Dam (28, 52). The uterine pigmentation which under the microscope is seen to extend to the phagocytic cells throughout the body is now under intensive study by Mason, who sees in it a possible clue to the essential vitamin E function (110).

**Influence on Teeth and Mucosa.**—An unlooked-for effect of tocopherol supplementation (15–50 mg. per day) in man is the decrease of deposition of tartar on the teeth. In 1942, we noticed accidentally that persons receiving extra tocopherols required much less frequent removal of tartar from the teeth. The matter was then tested professionally on eight patients and various dental clinics were informed.

Ten dentists in a certain area came to participate in a cooperative test which, for various reasons, including absences and additional work caused by the war, has not prospered. However, at The George Williams Hooper Foundation, San Francisco, the record is far different. During the past twelve months, Dr. Hermann Becks has used "vitamin E preparation in over 30 cases with extreme calculus formation. With the exception of four, we have seen very good results, especially in those patients that had recurrence of very hard incrustations within a period of two or three weeks following thorough subgingival curettage." The data are in course of publication.

In an endeavor to explain this phenomenon, we are reminded of the enhancement of phosphorus turnover in the vitamin E-deficient rat (165), and also of the experiments started at Northwestern University (17, 46) which showed that various quinones could control the activity of *Lactobacillus acidophilus* in the saliva. Of the quinones tried, only vitamin K could be considered naturally occurring. But there are two other classes of quinones available from diet: those which could derive from the phenolic bodies in peas and other vegetables which give such high spurious values for vitamin E by both Emmerie and Engel and Furter-Meyer tests; and vitamin E quinone itself. But if vitamin E quinone is present in traces in



the saliva, it should be present in fluids wetting other mucosa, such as the nose, sinuses, etc. If this should be the case, vitamin E might form one line of defense against those bacteria and viruses that are sensitive to quinones. This supposition is, of course, sheer speculation, but it forms the basis of experiments now in progress.

It is with some reserve that we mention vitamin E in connection with hay fever. During the past three years, Holmes has recommended massive doses of ascorbic acid (76), newspaper articles have advocated vitamin E *with manganese*, and Dam has thrown discredit on vitamin E by an experiment of one month's duration in which massive doses of *dl- $\alpha$ -tocopheryl acetate* were given (49). Holmes and Dam both erred in their dimensions, we believe, the doses being too large and the time of treatment too short. Considerable success has been achieved in preventing or minimizing the effect of hay fever by supplements of 25 mg. tocopherols and 50 mg. ascorbic acid taken daily for some months preceding and during the hay fever season. The favorable cases known to this laboratory do not constitute a medically attested record, but it is only by mentioning the matter that a proper test can eventuate.

### III. Requirements for Vitamin E and the Vitamin E Contents of Foods

The appraisal of these two dimensions is, next to discovering the fundamental vitamin E function, the most difficult task connected with this vitamin. Distinguished authorities (13) have dismissed vitamin E from their dietary calculations as "...not been established. If it is an essential nutrient for man the needs are not likely to present any major problem. . . ." This appraisal typifies the general attitude. Thus a column for vitamin E is missing from most dietary tables (97, 137). In recent summaries of vitamin requirements in pregnancy (32) and old age (152), two conditions calling especially for vitamin E, the vitamin is unmentioned or relegated to a cursory paragraph. There are plausible reasons for this neglect. Deficiency of this vitamin does not lead at once to a typical uncomplicated syndrome like scurvy, night blindness, or cheilosis. Consequently, there is no clear-cut response to vitamin E medication. Again, the changes that do occur mature so slowly that they often pass unrecognized. From a casual glance at the nutriture of the American people, it could be assumed that most persons have enough vitamin E for minimal needs. There are, however, two considerations that challenge this conclusion. One is the philosophical reflection that if there is ample vitamin E in the modern, partly processed diet, this vitamin must be the lone exception among all the rest! The other is concerned with the time element in vitamin E metabolism. An adult human contains about three to four grams of tocopherols distributed among various tissues and organs. His daily intake is generally less than 20 mg. These two quantities are evidently in

equilibrium under steady dietary conditions. If adjustment is dynamic and entirely unhindered, so that each *change* in the rate of tocopherol intake is immediately reflected in a proportional change in body stores, the rate of change should follow the usual logarithmic dilution law, whence:

$$\ln 2 \times \frac{\text{body stores}}{\text{daily intake}} =$$

period in days for half adjustment to changed vitamin E intake or, say:

$$H = 0.699 \times \frac{3500}{17.5} = 138$$

so that 138 days is the period, *H*, of half adjustment. However, the body stores are not in entirely free equilibrium with intake, but respond sluggishly. Part of the incoming vitamin makes abnormally high local concentrations in the gut and circulation and is metabolized or excreted without influencing the deeper seated depots. The effective therapeutic fraction, *q*, is therefore less than 17.5 mg., and the half-period is correspondingly greater—we estimate six months to a year. This means that even though the tocopherol intake may be adequate during one portion of a lifetime, if it should become inadequate later, it may take years for the effect to be manifest, and then the relation between cause and effect may pass unnoticed. With the implied connection between vitamin E and longevity (104), and the likelihood that diet and the utilization of diet alters adversely in later life, this aspect of the problem requires careful future examination.

**Vitamin E Requirements.**—Because of this long half-period and the difficulty of conducting controlled dietary experiments over extended intervals, the requirements for man have not been determined. Minimum requirements have been found for certain animals and the figures are sufficiently uniform from species to species (Table XII) to justify applying them to man as a first approximation and with careful interpretation. The minimum quantity referred to in Mason's paper (108) is, he informs us, a borderline prophylactic dose which protects most of the animals, most of the time, from most of the symptoms (sterility, dystrophy, pigmentation), but is not, with certainty, prophylactic for all symptoms in any given animal. The minimum certain dosage would be double the figures shown in Table XII, and the optimum would be about ten times as much. Again, the quantities have been determined from an arbitrarily selected diet. If the animals are supplied with more unsaturated fat their tocopherol requirements increase, and so on. The vitamin E standard used by Mason was *dl*- $\alpha$ -tocopheryl acetate. Equivalent borderline quantities for natural *d*- $\alpha$ -tocopherol would be 0.7 times and for natural mixed tocopherols about

1.2 times (allowing for the sparing action of the  $\gamma$ - for the  $\alpha$ -tocopherol) the figures in Table XII. These values should be doubled again for *safe* minimum quantities. As our present provisional estimate, we adopt 0.7 mg. synthetic *dl*- $\alpha$ -tocopherol per day per kg. body weight as the *safe minimum prophylactic dose* (S.M.P.D.), which will be taken as equivalent to 0.5 mg. *d*- $\alpha$ -tocopherol and 0.84 mg. of the mixture of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols that occurs in diet.

TABLE XII  
APPROXIMATE VITAMIN E REQUIREMENTS

Species	Symptom to be prevented	Estimated requirements, mg./day/kilo body wt.	Ref. No.
Rat	Sterility	0.33-0.6*	41, 42, 50, 106
	Muscular dystrophy	0.4-0.5	41
	Uterine pigmentation	0.3-1.0	118
Rabbit	Muscular dystrophy	0.3-1.0	37, 38, 113
Chicken	Encephalomalacia	1.0-1.2	124, 125
	Exudative diathesis	3.0	26
Sheep	Stiff-lamb disease	2.0	167
Duck	Muscular dystrophy	4-16	122

\* This value is for natural *d*- $\alpha$ -tocopherol whereas the other values are for synthetic *dl*- $\alpha$ -tocopheryl acetate.

Completely reliable analyses for the common foods, before and after cooking, are not yet available. Some items rich in the vitamin and low in carotenoids, such as the vegetable seed oils and wheat and corn germs, have probably been measured correctly. The vitamin E content of certain meats and vegetables will require minor revision. However, the data assembled by Emmerie and Engel and by Quaife *et al.* (Table XIII) enable the diets to be assessed with errors, probably far less than the discrepancies which will be demonstrated to exist between probable human requirements and the dietary supply. Very few of the food measurements have discriminated between  $\alpha$ - and  $\gamma$ -tocopherols. It will be assumed that the values in the literature refer to an equal mixture of the two and that the S.M.P.D. for a human adult weighing 70 kg. is 59 mg. daily, based on the ratio of the body weights of the rat and man.

There are other methods of calculation that lead to a lesser estimate. The vitamin E intake should be related to food intake as well as body weight. If this were not the case a large animal with a small food intake would find no natural food with enough vitamin E to support life, which is

clearly ridiculous. Again the tocopherol minimum requirement must vary with the relative proportions of protein, carbohydrate, and saturated and unsaturated fats in the diet. The problem is to maintain a tocopherol concentration in each diet appropriate to that diet. This raises the

TABLE XIII  
VITAMIN E CONTENT OF VARIOUS FOODS

Food	Total tocopherol,* mg./100 g.	Ref. No.	Food	Total tocopherol,* mg./100 g.	Ref. No.
<i>Cereals</i>			<i>Vegetables: (cont'd.)</i>		
Barley	3.2-5.2	35	Potatoes (boiled)	0.1	35
Bread (white)	1.4	35	Spinach	1.7	35
(brown)	2.1	35	Sprouts (Brussels)	1.7	35
Corn	10.0	35	Turnip	0.02	35
(white)	2.5	34	<i>Dairy products</i>		
(yellow)	3.1	34	Butter	2.1-3.3	35
	1.5-3.6	16		2.7	130
Doughnuts	2.0	130	Cheese (20% fat)	0.6	35
Oats	2.0	35	Milk (2.5% fat)	0.02	35
	5.0	34		0.11-0.13	130
Rice	0.4	35	<i>Meats + poultry products</i>		
Rye	2.2-3.5	35	Eggs (boiled)	3.0	35
Rye (flour) (60%)	2.0	36	(raw)	3.0 B	130
(bleached)	0.5	36	Meat	0.6	35
Soybeans	3.7	34	Beef	1.2 B	130
Wheat (whole)	2.6-3.4	35	Pork (chops)	0.77 B	130
	0.91	12	Rabbit	0.8	75
	3.7	34	<i>Fats and oils</i>		
(germ)	2.3-5.4	16	Cocoa fat	12.5	35
	27.0	36	Corn oil	250.0	35
	15.84	12		90.0	45
	15.9	34		103.5	81
(flour) (70%)	1.7	36	Cottonseed oil	82.5	81
(60%)	0.03	12		91.0	45
(bleached)	0.5	36	Olive oil	3.0	35
<i>Vegetables</i>			Lard	1.2-1.9	130
Beans (kidney)	1.2	35	Margarine	67.0-87.0	130
(white)	4.0	35	Peanut oil	26.0	35
Beets	0.2	35		36.5	81
Cabbage (white)	0.7	35		48.0	45
Carrots	1.5	35	Soybean oil	120.0	35
	0.33 B	130		92.3	81
	1.6	159		96.5	45
Celery	2.6	35	Wheat germ oil	150.0-250.0	35
Endive	2.0	35		274.0	81
Kale	8.0	35		190.0	34
Lettuce	0.6	35	Whole wheat oil	124.0	81
Onions	0.2	35			
Parsley	5.5	35			
Peas (green)	5.4-6.4	35			

\* Values represent total  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherol content except where followed by B (bioassay). The bioassay values represent  $\alpha$ -tocopherol.

question as to whether bread made without the wheat germ, salad oil and margarine prepared without the full tocopherol contents of the crude oils, fruit eaten without the core and the rind—and so forth—do, in fact, preserve a *tocopherol concentration* in the diet on which the human body can best operate.

Concerning the diet calculations, the adult rat weighing 300 g. eats about 15 g. of laboratory feed daily and, according to estimates made above, requires an S.M.P.D. of 0.25 mg. natural tocopherols daily. The human being eating 700 g. would require  $(0.25 \times 700)/15$ , or about 11.7 mg. of  $\alpha$ - and  $\gamma$ -tocopherols daily. This estimate is five times less than from body weight ratios. Instead of choosing between the two estimates, we prefer to combine them. A small person will require less vitamin E

\*  
TABLE XIV  
MINIMUM DAILY REQUIREMENTS FOR VITAMIN E\*

Alternative forms of vitamin E	Normal adult calculated from		Pregnant woman calculated from		Average in diet of industrial population (see Table XV)	Normal adult calculated according to formula†
	Body weight	Diet	Body weight	Diet		
Natural <i>d</i> - $\alpha$ -tocopherol	35 mg.	7 mg.	39 mg.	11 mg.	3-24 mg.	16 mg.
Synthetic <i>dl</i> - $\alpha$ -tocopherol	49 mg.	10 mg.	55 mg.	15 mg.		23 mg.
Equal mixture of natural $\alpha$ - and $\gamma$ -tocopherols	59 mg.	12 mg.	65 mg.	18 mg.		28 mg.

\* These requirements are predicted on a body weight of 70 kg. for a moderately active adult consuming 3000 Calories daily.

† Daily requirements in mg.  $\alpha$ - +  $\gamma$ -tocopherols =  $\frac{\text{kg. body weight}}{3.5} + \frac{\text{Calories, diet}}{375}$ .

than average to maintain an adequate concentration of tocopherol in the body. A large eater will excrete more tocopherols; an active worker will metabolize more. The formula which we have chosen for calculating minimum daily requirements takes into account body weight and calories but not the composition of the diet, and in this respect it is defective. The equation then becomes:

$$\text{mg. } \alpha\text{- + } \gamma\text{-tocopherols} = \frac{\text{kg. body wt.}}{3.5} + \frac{\text{Calories}}{375}$$

The denominators have been chosen, arbitrarily, to allow body weight calculations to contribute two-thirds and diet ratio, one-third, to the combined estimate. The data and conclusions from the various calculations are given in Tables XIV to XVI. The data are presented in micrograms and milligrams to allow the reader to follow the argument. It is *not* implied that physiological needs, either average or individual, can be predicted with this degree of accuracy.

**Vitamin E Adequacy of Various Diets.**—Seven diets (Table XV) have been compiled as representing persons in health and disease, active and sedentary habits, good and depressed circumstances. The food table and those rows in the diet table that list total tocopherol intake show that three apparently innocent preferences, as between butter and margarine, white and whole wheat bread, and ice cream and pie, have a tremendous influence on the tocopherol adequacy of the diet. In no case does the diet automatically insure sufficient vitamin E, though the active and well-to-do farmer is likely to receive sufficient, on the average. The elderly bachelor or spinster living in reduced circumstances (should we not say oxidized circumstances?), and the person on a smooth diet will certainly be deficient, unless conscious choice is made of items rich in vitamin E. Curiously enough, the moderately well-to-do family, income \$5000, is most consistently low in view of the calculated needs. The underprivileged Southerner, who has been demonstrated to have low plasma levels of vitamin E (63), nevertheless receives a considerable quantity if he eats *fresh* corn grits. This shows that the present survey fails to indicate which diets contain the best carriers, synergists, and cooperators (steps 8, 9, and 10) for vitamin E to insure proper health. One significant fact emerges, namely, the important place that vegetable fat, exemplified by margarine, occupies as a source of vitamin E. The average American appears to receive about 15 mg. tocopherols daily against a calculated requirement of 25 mg. Substitution of tocopherol-rich items can double the intake without requiring much adjustment in calories or money (Table XVI).

The subject covered by this argument will require long and complicated experimentation to arrive at an entirely satisfactory assessment. For instance, the figure adopted for the requirements of the rat is open to question. The diets of the test animals in the basic measurements made by Mason, Evans, and others were, from the very nature of things, abnormal for the animals. Casein, sucrose, salt mixture, and synthetic  $\alpha$ -tocopherol administered separately by dropper are not the dietary equivalents of lettuce, alfalfa, and cabbage, or whatever the animals ordinarily eat. The total utilization factors,  $\phi$  and  $\psi$ , must be different in the two cases. For our present calculation, the minimum animal requirements need re-evaluation by feeding the E vitamins in the form of natural foods, aliquots of which will be chemically analyzed for tocopherols to determine the actual dosage. However, the provisional conclusion remains that human diet is low in vitamin E. The calculations just given are typical of those that can be made for various farm animals, including the cow, the sheep, and the pig. Lack of space precludes their development here. In



<i>Butter or Margarine Sugar</i>	200 200 100	700 20,000	50 100	175	50 50	175 5,000	100					50 50	175 5,000	50 50	175 5,000
<i>Dinner</i>															
Soup	100													25	1,500
Fruit juice	400	1,000	50 300	750	100	250	200	175	300			25 150	375	75	
Meat															
Egg(s)															
Cheese															
Pork fat															
Potato	300	450	200	300	100	150						100	150	100	150
Vegetable (carrots, beets, etc.)	100	500	50	250	50	250	100	4,000						150	750
Greens															
Brussels sprouts															
Salad and dressing															
Onions			200	1,000										100	500
Crackers															
<i>Bread (white) or (brown)</i>	300 300	1,500 2,400	100	500	100	500						100	500	100	500
Biscuits															
Corn meal	300		300		200										
Dessert (ice cream or pie)	300	10,000	300	10,000	200	6,700	200	500 2,250				100 100 100	3,300	150 150	5,000
Jelly															
Fruit															
Milk	300	420	50	150	50							50	150	150	210
Cream	200	700	50	175	50	175						50	175	50	175
<i>Butter or Margarine</i>	200	20,000	100		50	5,000						50	5,000	50	5,000
Sugar												100			
Total Calories	5,050		2,925		1,350		1,450		1,945			1,925		2,175	
Upper tocopherol level, µg.		88,040		18,315		24,150		17,250					24,730		27,865
Lower tocopherol level, µg.		17,690		8,315		2,975		17,250					6,955		8,390
Calculated min. re- quirements, 70- kg. man, µg.		33,500		27,800		23,600		23,900					25,100		25,890



TABLE XVI  
VITAMIN E CONTENT OF A HYPOTHETICAL, BUT POSSIBLE, DIET

Meal	Calories	Tocopherols, $\mu$ g.
<i>Breakfast</i>		
Fruit juice	50	....
Eggs (2)	150	3,000
Bacon	150	300
Cereal (oatmeal)	100	875
Milk	150	210
Sugar	50	....
Coffee		
Sugar	50	....
Cream	50	150
Toast (whole wheat)	150	1,200
Butter	100	350
<i>Lunch</i>		
Soup	100	....
Sandwich		
Cheese	100	175
Bread (whole wheat)	150	1,200
Margarine	50	5,000
Pie	300	10,000
Coffee		
Sugar	50	....
Cream	50	150
<i>Dinner</i>		
Meat	300	750
Potatoes	100	150
Peas	50	3,000
Lettuce salad	100	500
Bread (whole wheat)	150	1,200
Butter	100	350
Pie	300	10,000
Coffee	..	....
<i>Total</i>	2,900	38,410

sum, however, the barn-fed cow is critically short. The sheep exhibits occasional deficiency in the form of stiff-lamb disease. Note that this condition is developed when the animal is receiving "rich natural" pasture diet. The pig, unless fed solely on stale garbage, appears to have sufficient, both by calculation and experiment. Fur-bearing animals bred in captivity, especially the fox and the mink, benefit from supplementation with vitamin E.

#### IV. Critique and Summary

In seeking to interpret all the physiological activities of the E vitamins, in terms of oxidation control, we have come squarely against the fact that

$\alpha$ -tocopherol has a more fundamental function, at present not understood. Nevertheless, the search for antioxidant effects has been fruitful. In this connection, we have developed a table of synergistic stages, or factorial steps, of vitamin utilization which has a very real meaning in the handling of vitamins, but, rather lamely, we have had to leave this almost entirely without quantitative illustration. Our computations of dietary sufficiency of vitamin E are inconclusive, but are certainly suggestive of a general American shortage. We have not dealt with the question of supplementary dosage and this we must now attempt.

There are, evidently, four types of vitamin E inadequacy—in the individual (1) who does not have enough in his diet, (2) who has temporarily increased needs, (3) who cannot absorb enough *from* his diet, and (4) who cannot utilize that which he absorbs. The first case is evidently the most common and will be taken care of at almost any income level by alteration of eating habits or a modest medicinal supplement of 10–15 mg. daily. The second applies chiefly to pregnancy, lactation, and old age, with possible implications in infancy. We set the optimum needs of the expectant mother at 50 mg. per day, or a supplement of 35 mg. The third, typified by the subject with celiac disease, will respond to larger doses, doses with bile salts, parenteral administration of water-soluble preparations, and so forth. By taking 100 mg. he will acquire perhaps 20 mg. The unsolved problem is the fourth type who is deficient in carrier, fixative, or coenzyme constituent. It is the present practice to treat these cases with massive doses which eventually, as Bicknell and Prescott point out (127), and we have attempted to rationalize, aggravates the very condition it attempts to cure. If the syndrome can be relieved by vitamin E, 200 mg. daily, or preferably 100 mg. daily over longer periods, would seem to be the maximum medicinal dosage. The recalcitrant cases, if involving tocopherol therapy at all, require oblique attack of the kind indicated by Milhorat and Bartels (115).

A number of anomalous dietary situations remain to be explained. The southern preclinic patient of Table XV has sufficient dietary tocopherol, but a low plasma content. The Eskimo receives enormous quantities of vitamin A and unsaturated fats but very little E, yet he lives and breeds. The Tibetan uses rancid fat daily in his tea; his cousin in lower Outer Mongolia is saturated with  $\gamma$ -tocopherol and vitamin C through soybean diet. What are their relative longevities? In spite of these unanswered questions, and in spite of its lesser synergistic role, which is more specific and less universal than we had supposed, the great practical significance of vitamin E demands attention. It is essential for tissue integrity and

the protection of body fat. It regulates the oxidative potential of the gastrointestinal tract. It is the balance wheel of the oil-soluble vitamins and the unsaturated fats. It is implicated in phosphorus metabolism and oral hygiene and its effects extend to the utilization of oxygen and the aging of the whole animal organism. Finally, its carelessly assumed sufficiency in the diet of humans and farm animals has led to its neglect in practical nutrition.

We close with a list of problems which appeal to us as being critical in vitamin E research, in order of their practical importance.

1. Development of reliable and specific analyses for  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tocopherols in food and tissue.
2. Accurate estimation of tocopherols in human and farm-animal diets.
3. Careful evaluation of human vitamin E requirements.
4. Complete amino acid analysis of normal and dystrophic tissue, of normal and cancer tissue.
5. Isolation of the biologically active enzyme or complex of  $\alpha$ -tocopherol.
6. Identification of the carrier and fixative agents for tocopherols in the animal body.
7. Measurement of the possible *in vivo* conversion of  $\beta$ - and  $\gamma$ - to  $\alpha$ -tocopherol.

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